CHARACTERISATION OF THE ANTI-VIRAL T-CELL RESPONSE INDUCED BY HUMAN CD137 LIGAND DENDRITIC CELLS

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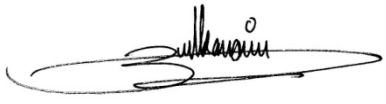
Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Dendritic cell (DC) vaccine is a potent form of therapy useful for the treatment of cancer. DCs are professional antigen-presenting cells (APCs) that are essential in regulating and orchestrating T-cell mediated immunity and this property has prompted intense research in the use of DC-based immunotherapy. However, DC vaccination is still in its infancy and improvements in terms of DC preparation, antigen-loading and increasing their immunostimulatory capacity are required. Here, we describe a potent and novel type of DCs generated by CD137 ligand (CD137L) reverse signalling into peripheral monocytes which have the capacity to induce superior antigen-specific T-cell response.

CD137L is expressed on peripheral human monocytes and delivers a potent activating signal via reverse signalling. Treatment of monocytes with a recombinant CD137 protein that induces reverse signalling through CD137L was reported to induce their differentiation to DCs that are capable of stimulating allogeneic naïve T cells to high perforin expression. In this study, we show that CD137L-generated DCs (CD137L-DCs) can be further matured leading to increased expression of DC markers such as CD83, CD86 and HLA-DR. This in turn enables a stronger activation of allogeneic T cells. We also tested the ability of CD137L-DCs to trigger antigen-specific T-cell activation in an autologous setting. Using cytomegalovirus (CMV) pp65 peptides, we show that CD137L-DCs are able to potently activate both $CD8^+$ and $CD4^+$ pp65-specific T cells to a level superior than T cells activated by conventional GM-CSF/IL-4 generated DCs (classical DCs). pp65-specific T cells restimulated by CD137L-DCs also secrete higher levels of IFN-γ and IL-13 than mature classical DCs which suggest their capacity to activate both

Th1 and Th2 subpopulations of $CD4^+$ T cells. Maturation of CD137L-DCs further potentiates this response. More importantly, these CD137L-DC-activated T cells are able to induce a stronger antigen-specific killing of HLA-matched target cells than T cells activated by classical DCs. To further characterise CD137L-DCs, their transcriptional profile was assessed. Through clustering and cMAP analyses, we determined that CD137L-DCs are more similar to mature classical DCs than to immature classical DCs or macrophages. Nonetheless, macrophage-specific genes such as podoplanin and CLEC5A were upregulated in CD137L-DCs suggesting a phenotypically different form of DC.

As a whole, *in silico* and *in vitro* data show that CD137L-DCs are phenotypically unique DCs with potent T-cell activating capacity and should be evaluated for human immunotherapy.

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LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ACT	Adoptive cell transfer
AICD	Activation-induced cell death
ALCAM	Activated leukocyte cell adhesion molecule
APC	Antigen presenting cell
APC	Allophycocyanin
BCC	basal-cell carcinoma
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDC	Classical DC
CFSE	Carboxyfluorescein succinimidyl ester
CLEC	C-type lectin domain family
cMAP	Connectivity map
CMV	Cytomegalovirus
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T-lymphocyte antigen 4
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Damage-associated molecular pattern
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DMBA	7,12-Dimethylbenz(a)anthracene
DMSO	Dimethyl sulfoxide
EBV	Epstein–Barr virus

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factors
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FDA	Food and Drug Administration
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing practice
GO	Gene ontology
HLA	Human leucocyte antigen
HNSCC	Head and neck squamous-cell carcinoma
HPC	Hematopoietic progenitor cell
HSPC	Hematopoietic stem and progenitor cell
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
ILA	Induced by lymphocyte activation
iMC	immature myeloid cell
Imm	Immature
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LC	Langerhans cell
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen-1
LIMMA	Linear model for microarray data
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal antibody

MART-1	Melanoma antigen recognized by T-cells 1
Mat	Mature
MCA	Methylcholanthrene
M-CSF	Macrophage colony-stimulating factor
MDSD	Myeloid-derived suppressor cell
MFI	Mean fluorescence index
MHC	Major histocompatability complex
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase
moDC	Monocyte-derived DC
NK	Natural killer
NKT	Natural killer T
NSCLC	Non-small cell lung cancer
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Programmed cell death
pDC	Plasmacytoid dendritic cell
PD-L	Programmed cell death ligand
PDPN	Podoplanin
PE	Phycoerythrin
PG	Prostaglandin
PHA	Phytohaemagglutinin
PI	Propidium iodide
poly (I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern-recognition receptor
PS	Penicillin streptomycin
R848	Resiquimod
RIN	RNA Integrity Number
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SMAC	Supramolecular activating complex
TAA	Tumour associated antigen
TAM	Tumour associated macrophage
TCR	T-cell receptor
Tfh	T follicular helper
TGF	Tumour growth factor
Th	T helper
TILs	Tumour infiltrating lymphocytes
TIRAP	Toll-interleukin-1 receptor domain-containing adaptor protein
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TNFRSF	Tumour necrosis factor receptor super family
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
VEGF	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.1. Cancer and the immune system

1.1.1. Overview on cancer immune surveillance

It is well established that the immune system combats pathogens such as microbes and viruses. Its function towards eliminating nascent transformed cells have, however, been heavily debated (Dunn et al., 2002). More recently, intense research by independent groups has provided overwhelming evidences on the existence of immune surveillance (Gao et al., 2003; Girardi et al., 2001; Street et al., 2002).

The concept of immune surveillance is based on the paradigm that the immune system tracks and detects anomalies developed by cells and tissues, and that before potential nascent tumours can form, these transformed cells are eliminated by the immune system. It is thought that a breach in the immune surveillance and subsequent immunological killing initiates the progression towards the formation of solid tumours and malignancies. Indeed, the identification of tumour antigens supported the notion of the immune system being able to differentiate between healthy and transformed neighbouring cells (Klein, 1966; Yang and Yang, 2005).

1.1.1.1. Evidences of immune surveillance

Immune surveillance is mediated by both the innate and adaptive arms of the immune system. Immunodeficient mice have been observed to have an increased carcinogen-induced tumour burden compared to their immunocompetent counterparts. Mice models depleted of innate NK and NKT cells had 2-3 times more methylcholanthrene (MCA)-induced tumour formation than the corresponding control mice (Smyth et al., 2001). Similar

results were observed in C57BL/6 mice depleted of only NK cells (Kim et al., 2007). Several elegant studies also provided compelling evidences on the immune surveillance contributed by the adaptive arm of immunity. Shankaran et al. genetically engineered $Rag2^{-/-}$ and $Stat1^{-/-}$ mice which were deficient for mature B and T cells, and downstream IFN-γ signalling, respectively. The group also cross-bred the mice to generate $Rag2^{-/-}$, $Stat1^{-/-}$ progeny. Mice that were $Rag2^{-/-}$, $Stat1^{-/-}$ were highly susceptible to MCA-induced tumourigenesis as 13 out of 18 mice developed tumours while this was seen in only 11 out of 57 wild-type mice. Mice that were either $Rag2^{-/-}$ or $Stat1^{-/-}$ had similar tumour incidence as $Rag2^{-/-}$, $Stat1^{-/-}$ mice, suggesting that B cells, T cells, and/or IFN-γ signalling are equally important in suppressing tumour development (Shankaran et al., 2001). Further, Girardi et al. confirmed the significance of functional T cells as TCR-β knockout mice, which lack functional $\alpha\beta$ T cells, were highly susceptible to DMBA-induced and TPA-induced carcinogenesis (Girardi et al., 2001).

Epidemiological studies also support the prevalence of immune surveillance in humans. Follow-up studies of transplant patients who were immunosuppressed or those with primary immunodeficiency demonstrated heightened preposition to development of cancer (Gatti and Good, 1971). Additionally, histopathological observation of human tumours provided clear indication of the presence of tumour infiltrating lymphocytes (TILs) and other immune cells in the tumour microenvironment (Busam et al., 2001; Holmes, 1985). Although certain immune cells in the microenvironment may be pro-tumour in nature, such as IL-10 and TGF-β producing tumour-associated macrophages (TAMs) and regulatory T cells (Tregs), there is a positive correlation between the presence of high-density tumour-specific $CD8^+$ T cells with good prognosis in various types of cancer such as colon cancer, lung

carcinoma, breast cancer and malignant melanoma (Haanen et al., 2006; Kawai et al., 2008; Naito et al., 1998; Yoshimoto et al., 1993). Again, the importance of $CD8^+$ T cells was highlighted by Pages and colleagues in a study on colorectal cancer. Patients with large mass of effector $CD8^+$ cytotoxic T lymphocytes (CTLs) together with effector $CD4^+$ T helper 1 (Th1) cells in the tumour corresponded to lack of metastasis and protection against tumour recurrence (Pages et al., 2005). However, in certain patients with high numbers of infiltrating lymphocytes, metastasis was still prevalent. Comprehensive phenotyping of TILs in a large cohort of patients revealed that protective T cells have a memory phenotype ($CD3^+CD45RO^+$) while metastatic patients generally lack these memory T cells (Camus et al., 2009). Therefore, both quantity (density) and quality (phenotype) of the tumour infiltrating T cells govern the overall tumourigenesis and possibly the outcome of the disease. Collectively, increasing data obtained from murine and human studies provide strong evidence for the existence of cancer immunosurveillance.

1.1.1.2. TILs and the tumour microenvironment

The abovementioned clearly illustrates the involvement of the immune system in the control of tumourigenesis. While different types of immune cells do infiltrate the tumour microenvironment, the key effector contributors have been described to be the NK cells and cytotoxic $CD8^+$ T cells. These two cellular subsets were initially designated as front line soldiers during infection and instigate the demise of infected cells; e.g. intracellular bacteria or virus infected cells (Sun and Lanier, 2011). Further investigation showed that these NK cells and $CD8^+$ T cells also display anti-tumour capabilities by directly exerting cytotoxic killing effects onto tumour cells, in both *in vitro* and *in vivo* situations in murine systems and this also occurs in humans (Budhu et al.,

2010; Kang et al., 2013; Koshy et al., 2013; Liddy et al., 2012). Even though many of these experiments performed required an additional adjuvant such as coating antigenic peptides onto tumours or redirecting tumour-specific $CD8^+$ T cells into the local tumour environment to instil clinical effects, there is no denying the tumour-suppressing potential of these immune effector cells.

Then why do malignancies still occur despite the existence of these defensive mechanisms? First and foremost, in hosts with spontaneously arising tumours, anti-tumour effector T cells by themselves are few and far between, and those that do arise are functionally incompetent thus reducing their ability to destroy tumour targets. Secondly, the tumour microenvironment is, unfortunately, a complex system encompassing inflammatory cells, that are both tumour-antagonising and tumour-promoting (Hanahan and Weinberg, 2011). Cells such as mast cells, neutrophils, B cells and M2-like macrophages (i.e. TAMs) are several tumour-promoting inflammatory cells that are often associated with neoplastic tissues lesions (Coffelt et al., 2010; DeNardo et al., 2010; Egeblad et al., 2010). Such infiltration of tumour-promoting cells leads to chronic inflammation and also to the release of epidermal growth factors (EGF), vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), among others, all of which facilitate tumourigenesis (Qian and Pollard, 2010).

More critically, a certain class of myeloid-derived cells in the tumour proximity has been regarded as direct inhibitors of tumour-antagonising NK cell and cytotoxic $CD8^+$ T cells. These myeloid-derived suppressor cells (MDSCs) are able to induce immune tolerance via direct and indirect means. An avenue of direct inhibition is to produce ROS and peroxynitrite which inhibits $CD8^+$ T cells by preventing T-cell-tumour interaction (Nagaraj et al., 2007). Secretion of immunosuppressive cytokines such as IL-10 and TGF-β act directly to

reduce the functions of anti-tumour $CD4^+$ Th cells and can also induce M2 macrophage polarization thus reducing IL-12 secretion required for Th1 responses (Sinha et al., 2007). IFN-γ production by NK cells are also negatively regulated by MDSCs via the blockade of NK cell activation receptor, NKG2D. This leads to NK cell anergy (Li et al., 2009). Indirectly, MDSCs can also down-regulate cell-mediated immunity by anti-tumour TILs via the expansion of Tregs (Serafini et al., 2008).

As a whole, coupled with the already low number of anti-tumour effector T cells in patients, MDSCs and other immunosuppresive cells in the tumour microenvironment virtually render anti-tumour T-cell response futile.

1.1.1.3. Inadequate anti-tumour T-cell numbers and function are contributed by aberrant dendritic cells

In order to generate a tumour-specific $CD8^+$ and $CD4^+$ T-cell response, tumour antigens need to be processed and subsequently presented to T cells by antigen-presenting cells (APCs). In normal physiological conditions, dendritic cells (DCs) are the most potent APC and are able to activate not only memory but also resting naive T cells, and are thereby critical components required for efficient mounting of an immune response. On the contrary, DCs in cancer patients are usually numerically infrequent, functionally aberrant and are not able to instigate efficient activation of tumour-specific effector T cells (Gabrilovich, 2004).

Many early studies have observed a low total number of functionally competent DCs in cancer patients such as those with head and neck squamous-cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC) and breast cancer (Almand et al., 2000; Della Bella et al., 2003; Wojas et al., 2004). Indeed, patients with early stage HNSCC had 2-folds lower amount of

DCs in their peripheral blood as compared to control healthy donors while further reduction of up to 4-folds was present in advanced tumour patients (Almand et al., 2000). Seemingly, the presence of larger tumours correlated with curtailed overall DCs as the surgical removal of tumours reconstituted the number of peripheral blood DCs (Hoffmann et al., 2002; Wojas et al., 2004). This is suggestive of a direct influence of tumours onto DC homeostasis. Overall, the repercussion of a reduced number of functional DCs is that anti-tumour T cells are not adequately stimulated and mobilised, leading to a progressing in tumourigenesis.

To initiate a DC response, apoptotic bodies or soluble antigens are engulfed, and this is followed by their activation (and therefore maturation) and migration to the secondary lymphoid organs where interaction with peripheral T cells occurs. Proper activation of DCs is an integral requirement as it arms DCs with co-stimulatory molecules such as CD80, CD86 and MHC class II, all of which provide necessary signals for adequate T-cell activation (Skalova et al., 2010). Nevertheless, on top of the sparse amount of DCs in tumours of cancer patients, DCs are usually phenotypically immature. No or low levels of CD80 and CD86 were previously detected in tumour-bearing DCs of patients with renal-cell carcinoma or prostate cancer (Troy et al., 1998a; Troy et al., 1998b). In basal-cell carcinoma (BCC), about 10 % of peritumoral DCs were either CD80 or CD86 positive and this value dropped to 1 % of intratumoral DCs (Nestle et al., 1997). Efforts to mature tumour-infiltrating DCs in rat models proved to be futile since the addition of exogenous GM-CSF and TNF or CD40L during in vitro cultures were not able to potentiate expression of either CD80 or CD86 (Chaux et al., 1997). This suggests that defective development of DCs starts from their precursors, rather than simply the antagonistic effect of immunosuppressive tumour microenvironment

contributing towards biologically abnormal DCs. Nevertheless, one cannot discount the immunosuppressive contribution by tumours as they are able to secrete anti-inflammatory cytokines such as IL-10 and TGF-β that recondition DCs and render them tolerogenic (Koido et al., 2010). Together, the lack of co-stimulatory molecule expression by DCs and their inability to be activated has detrimental consequences towards the initiation of an anti-tumour humoral and cell-mediated immunity. For instance, DCs isolated from colon cancer tissues and melanoma were not able to evoke potent T-cell proliferation and furthermore, primed T cells were anergic or induction of tolerance had occurred (Bonifaz et al., 2002; Chaux et al., 1997; Enk et al., 1997).

Another avenue utilised by tumours to induce DC dysregulation is through the means of inhibiting differentiation of immature myeloid cells (iMCs) into DCs by secreting tumour-promoting factors such as VEGF (Saito et al., 1998). In certain tumour-bearing individuals with lung or breast cancer, $CD33^+$ iMCs accumulate in peripheral blood in tandem with the decrease in DCs (Almand et al., 2000). These iMCs not only fail to differentiate into DCs but are by themselves immunosuppressive. Using an influenza-derived peptide model, iMCs were shown to inhibit IFN-γ production by peptide-specific $CD8^+$ T cells (Almand et al., 2001). Another study described a reduction in CD3ζ chain expression in T cells (CD3ζ is essential for the induction of intracellular signal-transduction pathways) in the presence of iMCs (Schmielau and Finn, 2001).

Collectively, aberrant DC development in cancer patients is predominant and negatively affects the T-cell mediated defence against neoplasm.

1.1.2. Harnessing anti-tumour T-cell mediated defence mechanism for immunotherapy

The failure to reject tumours by CTLs is attributed to immunosuppression and tolerance mechanisms such as the malfunction of DCs as previously described. A method to circumvent this effect would be to introduce a more vigorous CTL response (Melief and van der Burg, 2008). Various approaches are currently being used as immunotherapy tools to augment this reaction and these include T-cell adoptive transfer and DC-based vaccines. The former approach involves the isolation, *ex vivo* expansion and reinfusion of tumour-reactive T cells into patients. As previously noted, T cells have the ability to target and destroy tumours, however, such anti-tumour effects are more often than not dampen by the immunosuppressive tumour environment and also inefficient DC activation. Thus, the isolation of T cells from the tumour environment allows for their enrichment a more conducive *ex vivo* setting. Once a favourable number of tumour-specific T cells has been achieved, they can be infused back into the patient in combination with a lymphodepleting preparative regimen to deplete T cells and B cells (Restifo et al., 2012). This form of immunotherapy has been done with relative success in melanoma patients (Dudley, 2011; Rosenberg et al., 2011).

While adoptive T-cell therapy employs direct reconstitution of large numbers of antigen-specific T cells back into the patient, DC-based vaccines utilise the body's ability to mount their own T-cell-mediated response. DCs in cancer patients, especially those in late-stage disease, are systemically deregulated and may not be able to mount adequate T-cell responses. Thus, avenues such as to manipulate DC function, activation and antigen presentation are being studied to enhance the capacity of DC-induced T-cell activation which will enable a more sustainable and potent anti-tumour response. Currently,

three main approaches are being used as DC-based vaccines; (1) Non-targeted vaccines, (2) in vivo DC targeting and (3) vaccination using ex-vivo generated DC. Ongoing research and clinical trials on DC-based vaccines will be later discussed in section 1.3.4.

1.2. Dendritic cells

1.2.1. An overview

Dendritic cells (DCs) are key regulators of the immune system and their physiological properties allow them to serve as a crucial link between the two arms of immunity; that is, the innate and adaptive immunity. DCs continuously survey their environment for soluble antigens and foreign antigens, and if necessary, initiate and control an array of adaptive responses (Steinman, 1991). To do this, DCs acquire antigen material, process them and present the resulting peptide antigens to T cells and are therefore regarded as APCs. Their sheer efficiency to induce a primary immune response in resting naive T cells gives them the nickname of 'professional APC' (Steinman, 1991).

DCs were first discovered by the late Nobel Laureate Ralph Steinman in 1973. However, their unique role in the immune system was met with high scepticism. Forty years and countless discoveries later, we have gathered unequivocal evidence of DCs being an eminent constituent of the immune system; orchestrating the fine balance of immune function and regulation (Steinman, 2012).

1.2.2. Fundamentals of dendritic cell biology

DCs are of haematopoietic origin and are distributed widely in peripheral tissues. They express numerous pattern-recognition receptors (PRRs) which

sense danger signals via detection of pathogen-associated molecular patterns (PAMPs) in microbial pathogens or damage-associated molecular patterns (DAMPs), which are released by apoptotic or necrotic cells. Activation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), enables recognition of a class of pathogens and subsequently initiates an appropriate DC activation program (Iwasaki and Medzhitov, 2004). Besides, DCs are specialised to capture and process antigens *in vivo*, converting proteins into immunogenic peptides that are subsequently loaded onto major histocompatability complex (MHC) molecules to be presented to T cells (Guermonprez et al., 2002; Trombetta and Mellman, 2005). Upon acquisition of antigens in the periphery, tissue-resident DCs undergo activation and migrate to the secondary lymphoid organs such as the lymph nodes via afferent lymphatics where they interact with the circulating T cells to initiate clonal selection (Cyster, 1999; Itano and Jenkins, 2003; Randolph et al., 2005). Alternatively, lymph node-resident DCs can capture antigens which directly reach the lymph node (Itano and Jenkins, 2003). Essentially, DCs are able to recognise and select specific clones of naive T cells from a large repertoire of lymphocytes with distinct and unique antigen receptors.

The kind of response elicited is highly dependent on the type of antigen the DCs acquire. Self-antigens are presented by immature DCs, which are not subsequently activated, and therefore lead to immune tolerance through the activation of Tregs or suppressor T cells (Albert et al., 1998; Heath and Carbone, 2001). On the other hand, uptake of foreign (non-self) antigens often evokes clonal expansion of both antigen-specific $CD4^+$ Th cells and $CD8^+$ CTLs, though the exact response elicited is partly dependent on the subset of DC presenting the antigen and the maturation signals it receives (Banchereau and Steinman, 1998; Pulendran, 2005; Reis e Sousa, 2006).

Currently, three major signals dictated by the DCs are thought to contribute to the regulation of T-cell responses. The first is the presentation of antigenic peptide moiety on MHC molecules; known as signal 1. Expression of co-stimulatory molecules such as CD80, CD86, CD70, and OX40L are responsible for signal 2. Soluble factors such as cytokines secreted by DCs contribute to signal 3 and these factors influence the differential development of both $CD4^+$ and $CD8^+$ T cells (de Jong et al., 2005; Kalinski et al., 1999; Nestle et al., 1998b). Working in concert, these signals drive cell division and differentiation of $CD4^+$ T cells, giving rise to polarised subsets of effector cells. Th1 and Th2 are the best defined subsets, characterised by their production of interferon-γ (IFN-γ) and interleukin-4 (IL-4), respectively (Mosmann et al., 1986). More recently, pro-inflammatory IL-17-secreting Th17 cells, Tregs that can dampen effector function of other lymphocytes as well as T follicular helper (Tfh) cells that are specialised B cell helpers have been described as additional effector $CD4^+$ T cells (Harrington et al., 2005; Lund et al., 2008). In addition to their influence on cell-mediated immunity, DCs are also involved in humoral immunity by direct contact with B cells to present unprocessed antigens or via Th2 and Tfh cells (Bergtold et al., 2005; Qi et al., 2006). Furthermore, DCs also interact with NK cells of the innate immune system (Fernandez et al., 1999; Lucas et al., 2007).

1.2.3. Subsets of dendritic cells

1.2.3.1. Murine dendritic cells

DCs are heterogeneous and many subtypes have been described in mice. Historically, research had been done mainly on the splenic and lymphoid tissue DCs however, much attention is now focused on the non-lymphoid tissue counterparts such as skin and lung DCs (Helft et al., 2010).

DCs in the spleen are classified into $CD8^+CD11b^-CD4^-$ ($CD8^+$ DCs), $CD8^-CD11b^+CD4^+$ ($CD4^+$ DCs) and $CD8^-CD11b^+CD4^-$ (double negative DCs) (Vremec et al., 2000). The double negative DCs also predominate in the lymph node while Langerhans cells (LCs) predominate in the skin (Henri et al., 2001). In the non-lymphoid tissue, DCs are broadly divided into $CD103^+$ and $CD11b^+$ DCs while in the lamina propria, an additional subset, $CD103^+CD11b^+$ double positive DCs, is found (Bogunovic et al., 2009; Ginhoux et al., 2009). $CD103^+$ DCs have been described as the non-lymphoid tissue counterpart of lymphoid tissue $CD8^+$ DCs, in which both subsets are specialised in cross-presentation; a feature whereby exogeneous antigens are processed and presented onto MHC class I (Ginhoux et al., 2009). Other subsets found in mice include plasmacytoid DCs (pDCs) and inflammatory DCs which are characterised by MHC-$II^+CD11c^{lo}CD4^+$ and MHC-II^+CD11c^{int} $CD11b^+$ phenotypes respectively.

1.2.3.2. Human dendritic cells

Human DCs can be broadly divided into lymphoid- or myeloid-derived DCs. The former consist of pDCs which are defined by the surface expression of CD303 (aka BDCA2), CD304, ILT7 and CD123 (Colonna et al., 2004; Dzionek et al., 2000). While resting pDCs are involved in tolerance, their activation in response to viral infection, induces the production of type I interferon and their large stores of MHC class I molecules suggest a subsequent rapid $CD8^+$ T-cell response (Di Pucchio et al., 2008; Liu, 2005; Siegal et al., 1999). Thus, pDCs have been suggested to be involved in front-line anti-viral defence.

Myeloid-derived DCs are predominant and are widely distributed throughout the body. These DCs are generally categorised as $CD141^+$ DCs, $CD1c^+$ DCs

or $CD14^+$ DCs. Myeloid $CD141^+$ DCs and $CD1c^+$ DCs are found in lymphoid tissues, non-lymphoid tissues and blood. In non-lymphoid tissues, $CD141^+$ DCs express high levels of CD141 and are designated as $CD141^{hi}CD14^-$ $CD11b^{lo}CD11c^{lo}$ (Haniffa et al., 2012). These DCs are regarded as the homologues of $CD8^+$ DCs in mice as both subsets have a high capacity to cross-present antigens and activate naive $CD8^+$ T cells *in vitro* (Bachem et al., 2010). $CD1c^+$ subset DCs are also defined by the expression of CD11c, CD13, CD33 and CD172 (MacDonald et al., 2002; Mittag et al., 2011). These DCs constitute about 1 % of mononuclear cells in the blood, and in non-lymphoid tissues they are the main DC subset during steady-state. $CD14^+$ DCs are the third subset and are found only in tissues (lymphoid and non-lymphoid) but not in the blood. These dermal DCs are involved in humoral responses and can induce antibody-secreting B-cell differentiation as well as naive T cells to Tfh cells (Klechevsky et al., 2008; Matthews et al., 2012). Intriguingly, $CD14^+$ DCs are functionally and phenotypically intermediate of DCs and monocytes/macrophages and this population has been described to be recapitulated by peripheral blood $CD14^+$ monocytes (Chu et al., 2012; Penel-Sotirakis et al., 2012). Together, $CD1c^+$ DCs and $CD14^+$ DCs are the two main DC subsets located in the dermal region of the skin and this is in addition to epidermal LCs (Nestle et al., 1993).

The abovementioned DCs are present in steady-state during normal physiological conditions. Inflammatory DCs, on the other hand, only appear during inflammation or upon microbial insult and are considered as non-conventional DCs, (Liu and Nussenzweig, 2010). An example of such DCs are the TNF and iNOS-producing Tip-DCs which are phenotypically $CD14^-$ $CD1c^-$ (Zaba et al., 2009). During inflammation or infection, the population of inflammatory DCs is substantially heightened within a short time-frame and

this is thought to be contributed by peripheral blood monocytes which have been shown in mice to be able to differentiate into inflammatory-like DCs *in vivo* (Cheong et al., 2010). Nevertheless, their origin and potential role in inflammation are still unclear and are currently being investigated.

1.2.4. Monocyte-derived dendritic cells for DC-based immunotherapy

Monocyte-derived DCs (moDCs) are also designated as non-conventional DCs given the fact that they do not give rise to resident DCs in various lymphoid and non-lymphoid tissues in physiological steady-states (Liu and Nussenzweig, 2010). Owing to favourable factors, this subset of cells is considered ideal for DC-based immunotherapy. Human peripheral blood monocytes can be easily acquired by leukapheresis in large amounts and hence can be a useful source for generating DCs for use in vaccines (Wolf et al., 2005). Biologically, monocytes can be easily differentiated into immature DCs simply by the addition of exogeneous GM-CSF plus IL-4 (Banchereau et al., 2000). The resulting moDCs are highly phagocytic and therefore are able to take up soluble antigens and migrate to the draining lymph nodes. More importantly, this DC subset can be easily activated and matured by using various combinations of TLR-agonist and/or cytokine cocktails thereby improving subsequent T-cell activation (Rissoan et al., 1999). These characteristics are key factors that make moDCs an attractive tool for DC-based vaccine therapy. In the next section, we will discuss the use of such DCs as an attractive tool for cancer immunotherapy.

1.3. Cancer immunotherapy

1.3.1. An overview

Immunotherapy utilises the immune system to mount a response as a treatment against cancer. Immunotherapeutic protocols can be applied individually but are often used in combination with traditional treatments such as chemotherapy and radiotherapy. An array of strategies is currently being implemented to harness and evoke specific immune responses and these include the use on immunomodulatory antibodies, adoptive cell transfer and cellular vaccines (Alatrash et al., 2013). Although much will be reviewed on the current understanding and development of DC-based cellular vaccines, immunomodulatory antibodies and adoptive cell transfer approaches may be applied in parallel with cellular vaccines and will therefore be briefly discussed.

1.3.2. Immunomodulatory antibodies

Antibody-based anti-cancer therapy has been used to relative success in a number of clinical trials and there are currently 13 FDA-approved antibodies for various oncological applications (Sliwkowski and Mellman, 2013). Recent developments have allowed the generation of humanised monoclonal-antibodies (mAbs) that are non-immunogenic in humans and this has propelled wide spread clinical-based research to be endowed upon (Clynes, 2006).

Antibodies can function to kill tumour cells in a variety of mechanisms and are broadly divided into two groups; (1) direct tumour cell killing and (2) immune mediated tumour cell killing (Scott et al., 2012). In the former, agonistic antibodies can directly bind to certain tumour-associated receptors therefore

leading to activation and downstream proapoptotic mechanism. Examples of mAbs associated with this mechanism are Rituximab and Alemtuzumab which interact with CD20 and CD52, respectively in B cell leukaemia (Weiner, 2010). Other mAb such as ^{90}Y-labelled ibritumomab tiuxetan can deliver targeted radioisotopes to $CD20^{+}$ non-Hodgkin lymphoma and induce direct the demise of tumour cells (Cheson and Leonard, 2008).

Immune mediated tumour cell killing, as the name suggests, requires the association with various types of immune effector cells. Depending on the approach taken, T cells, macrophages, NK cells and complement pathway, amongst many others, are involved in this process. For instance, agonistic anti-CD137 mAb are able to co-stimulate T cells and demonstrate an anti-tumour efficacy in phase I clinical trials in melanoma patients (Molckovsky and Siu, 2008). Other mAbs bind to tumour antigens which lead to opsonisation and subsequent phagocytosis by macrophages or induction of complement pathways (Scott et al., 2012). MAbs that function as checkpoint blockade are also being actively studied in clinical trials. CTLA-4 is a negative regulator on T cells and acts as a checkpoint to prevent overstimulation. Thus, using antagonistic anti-CTLA-4 mAb such as tremelimumab or ipilimumab serves to ignore this checkpoint, allowing for prolonged T-cell activation (Hodi et al., 2010). The success of using this strategy has brought about further interest in developing mAb for checkpoint blockade. For example, PD-1 is expressed on activated T cells and upon engagement by its ligand, PDL-1 on APCs, induces apoptosis. Early clinical trials on PD-1 blockade have shown promising responses in patients with melanoma, colorectal cancer and renal cell carcinoma (Brahmer et al., 2010).

1.3.3. Adoptive cell transfer

The first report describing the use of adoptive cell transfer (ACT) was in 1988 in which Rosenberg and colleagues demonstrated the regression of tumours using TILs for immunotherapy on patients with metastatic melanoma (Rosenberg et al., 1988). Since then, much research has been undertaken to optimise various protocols and strategies in order to boost clinical efficacy.

ACT requires the identification of tumour-reactive TILs in excised tumour mass, *ex vivo* expansion, followed by reinfusion of large number of cloned TILs back into the cancer patient. Being an *ex vivo* procedure, it has the advantage of being able to be pre-selected for tumour-specific T cells (both $CD8^+$ and $CD4^+$) with high avidity and expanded in an environment free from immunosuppressive factors (Morgan et al., 2006). A recent completed phase II clinical trial (NCT00314106) on metastatic melanoma showed clinical benefits when lymphodepletion prior to autologous TIL transfer plus IL-2 treatment was administered to patients refractory to standard therapies (Dudley et al., 2008).

Activation of T cells is MHC-restricted and is therefore logistically laborious for large-scale therapy. To alleviate this drawback, T cells are being engineered to express chimeric antigen receptors (CARs) whose activation is not MHC-restricted but dependent on direct interaction with tumour antigens. CARs consist of an extracellular domain that recognise antigens and an intracellular co-stimulation activator domain such as those of CD28 and CD137 origin (Zhang et al., 2013). Such engineered T cells are currently in clinical trials as an alternative to autologous natural T-cell ACT. Other lymphocytes that are presently in use for ACT include NK and NKT cells.

1.3.4. Dendritic cell-based immunotherapy

1.3.4.1. An overview

As earlier mentioned, DCs are crucial mediators that initiate the adaptive arm of immunity including the activation of antigen-specific naive T cells. Their properties have prompted DCs to be designated a natural adjuvant for antigen delivery and aptly enough, are currently being exploited as an immunotherapeutic tool. A quick search on clinicaltrials.gov generated 289 clinical trial studies (as of 15th December 2013) that have been or are currently being undertaken, and this clearly indicates the keen interest in the pursue of DC-based vaccines. Presently, there are three main regimens for DC-based vaccines. The first two groups exploits endogeneous DCs; (1) non-targeted vaccines and (2) *in vivo* DC targeting vaccines, while (3) *ex vivo* generated DC vaccines, as the name suggest, requires DCs to be generated in a laboratory setting. Emphasis will be subjected to the latter mode of vaccination (figure 1).

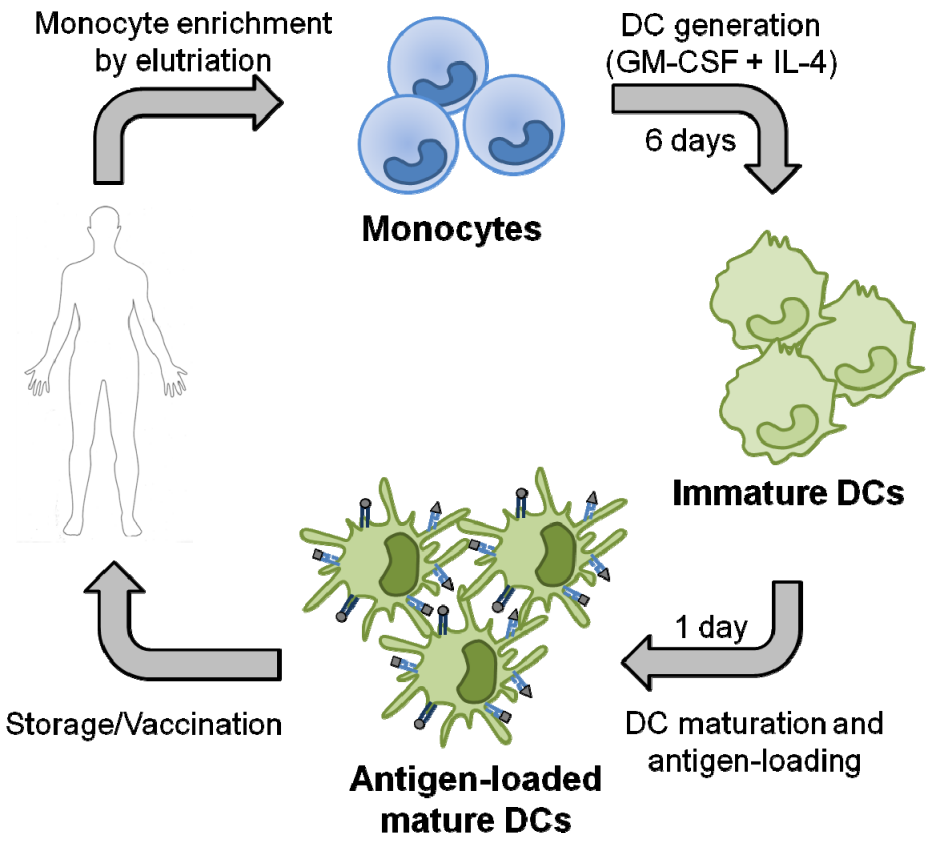


Figure 1. **Overview of *ex vivo* dendritic cell vaccine protocol.**

1.3.4.2. Conventional vaccination

Conceptually, conventional vaccinations are non-targeted and focus on loading endogenous DCs with antigens, or by manipulating certain aspects of tumours or the immune system to improve DC efficacy.

Early development using tumour-derived peptide antigens were focused on eliciting $CD8^+$ T cells using short 8-9 amino acid (aa) sequences (Boon et al., 2006). Although it was successful in propagating the effector T cells, the lack of $CD4^+$ Th1 response meant that using such peptides was not optimal to bring about potent CTL and associated memory cell responses (Filipazzi et al., 2012). Longer peptides that contains multiple epitopes that can activate both $CD4^+$ and $CD8^+$ T cells are the current mainstay and have been shown to be clinically effective in patients with high-grade vulvar intraepithelial

neoplasia (Kenter et al., 2009). More personalised protocols are also presently being explored whereby instead of using a pre-existing marker for a certain tumour, peptides representing antigens from an individuals' tumour are used as vaccines.

Other avenues of non-targeted vaccines encompass the usage of viral entities. Oncolytic viruses that preferentially infect tumour cells and destroy them can be modified to express GM-CSF in the hosts. This growth factor will in turn congregate DCs to the tumour microenvironment thereby increasing the likelihood of antigen uptake ensuing T-cell activation in the lymph nodes (Russell et al., 2012). Caution has to be taken as the tumour microenvironment is bathed in anti-inflammatory factors and therefore a parallel regime to reduce the immunosuppressive effects is often required. Additionally, addition of adjuvants to mature and polarise *in vivo* DCs are also necessary.

1.3.4.3. *In vivo* dendritic cell targeting

An array of surface proteins are expressed specifically on DCs and by targeting these markers, antibodies conjugated with tumour antigens can be delivered directly to the DCs. One of the pioneering investigation was to target DEC205$^+$ DCs *in vivo* (Hawiger et al., 2001). However, antigen-specific tolerance prevailed thus suggesting the need to administer DC maturation agonists such as those activating TLR3 or CD40. Indeed, the presence of such adjuvants skewed the response towards a more potent anti-tumour CD4$^+$ T-cell and CD8$^+$ CTL activation when CLEC9A was targeted using antibody-tumour antigen conjugate (Joffre et al., 2010). Alternative DC markers that can be targeted are DC-SIGN and CD40, as recent evidence

has suggested that targeting the latter leads to efficient cross-presentation correlating to strong $CD8^+$ T-cell responses (Chatterjee et al., 2012).

It is therefore apparent that the desired anti-tumour T-cell response can be predominantly acquired if the right DC-specific marker is targeted together with the addition of an ideal adjuvant. However, further appreciation of endogenous DC subsets in humans to determine the best candidate DCs to be targeted is required to further refine the clinical efficacy when opting for *in vivo* DC targeting therapy.

1.3.4.4. *Ex vivo* generated dendritic cells

Generating DCs in a laboratory setting has its benefits as they can be propagated in an immunosuppressive-free environment and grants greater possibility for DC manipulation (Schuler, 2010). The aim here is to cultivate large amount of potent DCs that can subsequently prime robust anti-tumour-specific T cells *in vivo* upon their reinfusion into patients (figure 1).

Several subsets of DCs and their precursor cells are being exploited for this purpose and an example are the peripheral blood pDCs. Early studies in metastatic-melanoma patients were promising as tumour-peptide pulsed pDCs were able to activate both $CD4^+$ and $CD8^+$ antigen-specific T cells (Tel et al., 2013). Alternatively, $CD34^+$ hematopoietic progenitor cells may be applied as a DC precursor although their usage in clinical trials are few and far between owing to a more challenging production process (Ueno et al., 2010). In 2010, Sipuleucel-T (tradename: Provenge^{TM}) received FDA-approval for treatment of prostate cancer, a first of its kind for DC-based vaccines. Enriched blood APCs, including DCs, were exposed to a fusion protein of GM-CSF and prostatic acid phosphatase antigen before being

reinfused back into patients. Overall survival was improved by ~4 months (median) and the risk of death was reduced by ~25% as referenced to control group (Higano et al., 2009).

Nevertheless, due to the ease for isolation and their availability in large quantities, $CD14^+$ peripheral monocytes are often used as precursors to develop autologous *ex vivo* DCs. Most clinical trials employ the standard culture protocol using GM-CSF plus IL-4 for a period of 2-6 days to generate immature moDCs followed by their exposure to maturation factors such as the Jonuleit cocktail (TNF, IL-1β, IL-6 and PGE2) to induce full DC activation (Erdmann and Schuler-Thurner, 2010; Jonuleit et al., 1997). This step is critical as different combinations of DC activating factors can determine the later polarisation of antigen-specific T-cell response and therefore the eventual clinical outcome. Next, antigens in various forms such as peptides, soluble proteins and whole tumour lysates, and more recently, transfection of mRNA encoding for defined tumour antigens are loaded into DCs to educate them for the desired antigen-specific T-cell response (Erdmann and Schuler-Thurner, 2010; Van Nuffel et al., 2012).

The convenience of utilising moDCs translated to widespread research and clinical trials involving this DC subset. Table 1 summarises some of the recent or ongoing clinical trials that involve *ex vivo* generated DCs. Many of these trials were able to show immunogenicity however, the discrepancy between the blood immune response and the rate of objective clinical response is all too obvious (Palucka and Banchereau, 2012). For instance, a study by Soleimani et al. on metastatic renal-cell carcinoma showed that upon vaccination of mature moDCs pulsed with autologous tumour lysate, specific T-cell sensitization was acquired as determined by the increased IFN-γ secretion and proliferation. However, none of the 14 vaccine-recipients

obtained complete remission (Soleimani et al., 2009). This clearly highlights the need for further refinement of DC-based protocols, whether in terms of DC generation, maturation, antigen loading or route of DC re-infusion. Also, combinational treatment aimed to reduce immunosuppressive effects or utilising DC vaccines in tandem with traditional radiotherapy and/or chemotherapy can be tested for their overall treatment efficacy.

Table 1: Examples of clinical trials using *ex vivo* generated monocyte-derived DCs (Adapted from Palucka et al., 2012)

Vaccine & antigen	Indication	Key observations	Reference
GM-CSF + IL-4 DCs with or without HLA-A*0201-restricted peptides or peptides alone	Metastatic prostate cancer	One of the first studies that tested the immunogenicity of DCs	(Murphy et al., 1996)
GM-CSF + IL-4 DCs with peptides, tumour lysates or autologous tumour-eluted peptides	Stage IV melanoma, renal cell carcinoma and malignant glioma	• Loading DCs with complex antigen preparations • Objective clinical responses	(Holtl et al., 1999; Nestle et al., 1998a; Yu et al., 2001)
Mature GM-CSF + IL-4 DCs and peptides	Stage IV melanoma	• Well-controlled and validated vaccine manufacture process • Testing mature DCs • Immunogenicity • Objective clinical responses	(Thurner et al., 1999)
GM-CSF + IL-4 DCs and tumour lysates	Refractory paediatric solid tumours	• Immunogenicity • Objective clinical responses	(Geiger et al., 2001)
Monocyte-derived DCs	Melanoma	Route of DC administration affects T-cell activation, with intra-dermal administration showing better responses than intra-nodal administration	(Lesterhuis et al., 2011)
Type1-polarised monocyte-derived DCs	Glioma	Combination of DC vaccination with polyICLC to trigger systemic inflammation driven by type I interferon family members	(Okada et al., 2011)

1.3.4.5. Maturation and development of type 1, 2 and 17 dendritic cells

DCs are immunomodulators and can not only mediate pro-inflammatory conditions but may also skew immune responses towards tolerance. Evidence has suggested that this is dependent on the activation status and that immature or partially-mature DCs are prone to inducing tolerance via expansion of both allogeneic and autologous antigen-specific Tregs (Albert et al., 2001; Dhodapkar et al., 2001; Jonuleit et al., 2000). On the other hand, DCs pre-activated with a variety of cytokines could polarise naive $CD4^+$ T cells towards a predominantly Th1 phenotype while $CD8^+$ T cells were highly activated as evidenced by high IFN-γ release (Jonuleit et al., 1997). Essentially, competent anti-tumour effects are synonymous with strong Th1 and $CD8^+$ CTL response and that has prompted a plethora of explorations to determine the conditions required to optimally activate DCs. IL-12 is an important factor secreted by DCs which has positive implications on Th1 development (Hsieh et al., 1993). Therefore, much is being focused on designing maturation cocktails that can propel IL-12 production.

The Jonuleit cocktail was one of the pioneering cocktails developed for maturation of human moDCs (Jonuleit et al., 1997). Despite the absence of IL-12 upon DC activation, it still led to a predominantly Th1 and $CD8^+$ CTL response. This fully cytokine-based cocktail consists of IL-1β, IL-6, TNF and PGE2. The first three are known pro-inflammatory cytokines while PGE2 supports overall growth of DCs and also increases CCR7 that allows for their migration to the lymph node: a character that is desired in a DC-based vaccine (Bruckner et al., 2012). This cocktail is widely used in many clinical trials and although it is considered the current gold-standard, other cocktails are being developed to refine DC activation especially in terms to increase IL-12 expression (Skalova et al., 2010). Kalinski and colleagues derived a

cocktail combining cytokines with TLR-ligands as IL-12 has been shown to require at least 2 'danger' signals for its production. Using poly (I:C), a TLR3 agonist, together with inflammatory cytokines IFN-γ, IFN-α, IL-1β and TNF, they were able to conjure potent IL-12 production (Mailliard et al., 2004). DCs were fully activated based on surface marker expression and were able to instil potent antigen-specific CTLs. However, a drawback was a decrease in their migration capacity as compared to DCs matured by Jonuleit's cocktail. Interestingly, a study by Zobywalski determined that although Kalinski's cocktail was able to fully mature DCs, their viability was compromised leading to lower yields during harvesting (Zobywalski et al., 2007). His team also proposed that instead of using poly (I:C), the TLR7/8 agonist, R848, should be used as it does not hinder expression of antigens inserted through exogenous RNA electroporation.

The preceding research was motivated by the desire to produced type-1 DCs that are expected to initiate Th1-mediated immunity, suggested to be integral for anti-tumour effects. Using different combinations of maturation factors, DCs can also be activated towards type-2 or type-17 phenotype (pro-Th2 and Th17, respectively), (Cintolo et al., 2012). Although classically associated with anti-parasitic defence and allergic reactions, Th2-mediated immunity stimulated by type-2 DCs may also have an anti-tumour role. It was found that Th2-mediated immunity initiated eosinophil influx into tumours and was able to clear lung metastasis of B16 melonoma in C57Bl/6 mice (Mattes et al., 2003). This anti-tumour effect was later shown to be dependent on Th2-cell release of the eosinophil chemokine, eotaxin. More recently, Th17 cells were identified as a separate helper T-cell subset (Harrington et al., 2005). Differentiation of IL-17 secreting T cells is dependent on IL-23 production by DCs that in turn require TLR signalling via TLR2, TLR4 or TLR7/8. There is

currently no consensus on whether Th17 are tumour-promoting or antagonising as evidence supporting either argument does exists. Still, Th17 cells demonstrated effective tumour regression in B16 melanoma mouse models (Muranski et al., 2008). Therefore, alternative modes of maturation should be carefully explored in order to exploit desired anti-tumour characteristics from differentially activated DCs. Instead of focusing solely on generating potent Th1 responses, alternative activation protocols that ultimately propel DCs to optimally induce widespread anti-tumour effects should be the key issue of future studies.

1.3.4.6. Delivering immunodominant tumour antigens

A critical but often challenging factor in designing DC-based vaccines is to identify effective immunogenic antigens and the approach for antigen delivery. Most tumour antigens are derivatives of self-antigens and may either be mutated or overexpressesed in tumours thus making them less immunogenic than non-self antigens. Therefore, an important criterion is to select tumour epitopes that will elicit a sufficient tumour-specific T-cell response.

Currently, peptides are the main form of antigens for DC-based vaccines. Short peptides such as those derived from melanoma MART-1/MelanA are often pulsed onto DCs to successfully elicit antigen-specific $CD8^+$ T-cell responses (Fay et al., 2006; Oshita et al., 2012). These 8-10 aa long peptides are however HLA haplotype-restricted and will only be immunogenic in the specific population harbouring the haplotype. Additionally, $CD4^+$ Th responses cannot be elicited thus reducing synergistic effects that are beneficial for overall clinical efficacy. Thus, current peptide design concepts are being redirected towards acquiring peptides that can evoke stronger $CD8^+$

T-cell responses, and at the same time bring about sensitisation of the $CD4^+$ T cells (Palucka and Banchereau, 2013). In order to heighten efficiencies in activating $CD8^+$ CTLs, Chauvin and colleagues utilised long peptides (26 aa) of MART-1/MelanA. The peptides were also modified at position 27 (A27L) which is known to be an essential residue for the anchorage of peptide to MHC class I (Chauvin et al., 2012). The elongated peptide coupled with a more robust peptide-MHC interaction translated towards a favourable increase in $CD8^+$ T-cell activation. Peptides that can elicit $CD4^+$ T cells are also available albeit less common. However, the use of combination of peptides or peptides pools (usually 15-mer peptides with 11 aa overlap covering the entire immunodominant antigen) is an attractive option as both $CD4^+$ and $CD8^+$ T cells can be stimulated (Lachmann et al., 2012). Furthermore, employing peptide pools can circumvent the issue of HLA-restriction.

Other researchers are also exploring novel methods to present tumour peptides onto DCs. This includes the use of whole tumour lysates as a source of antigen, RNA transfection into DCs to artificially induce expression of immunogeneic tumour proteins and fusion of DCs with autologous tumour cells (Koido et al., 2013; Nair et al., 2002; Nestle et al., 1998a; Salcedo et al., 2006).

1.3.4.7. Future direction for DC-based vaccines

Ultimately, the goal of DC-based vaccines is to instruct the immune system to generate an anti-tumour response. Although DCs can contribute to the innate arm of immunity by activating cytotoxic NK cells against tumours, the T-cell mediated defence is the predominant mechanism. Thus, future DC-based vaccines should be able to elicit (1) potent $CD8^+$ CTLs, (2) $CD4^+$ Th cells

which complement $CD8^+$ T-cell function and (3) effector T cells that are able to escape from the hostile tumour microenvironment and induce the demise of the tumours (Palucka and Banchereau, 2013).

At present, the conditions required to create a robust DC phenotype, capable of satisfying all three criteria has eluded scientists. Alternative avenues for DC preparation, maturation, antigen-loading and route of immunisation need to be thoroughly investigated. For example, two independent laboratories previously discovered a novel method to generate potent DCs by stimulating CD137 ligand signalling into peripheral monocytes (Ju et al., 2009; Kwajah and Schwarz, 2010). These DCs were found to be excellent activators of both $CD4^+$ and $CD8^+$ T cells in allogeneic settings. Riding upon this finding, this thesis will further characterise these novel DCs.

1.4. CD137 receptor-ligand system

1.4.1. Biology of CD137

There are currently nearly 30 members of the tumour necrosis factor receptor super family (TNFRSF) and a member that has recently earned much attention is the co-stimulatory molecule CD137. This receptor is also known as *induced by lymphocyte activation* (ILA), 4-1BB and is officially designated as TNFRSF9. Members in this family are characterized by the cysteine-rich pseudo-repeats in the extracellular domain (Mallett and Barclay, 1991). CD137 exists in two forms; either as a type I transmembrane glycoprotein on cells, or as a soluble protein produced via mRNA splicing (Michel et al., 1998; Schwarz et al., 1993; Setareh et al., 1995).

The initial discovery of this molecule was made more than two decades ago when Kwon et al. identified an activation inducible protein in murine T cells

and termed it 4-1BB. Its human counterpart was later independently identified by Schwarz et al. in activated T cells and was designated as ILA due to its nature of expression (Schwarz et al., 1993). CD137 is located on chromosome 4 and chromosome 1p36 in mice and humans, respectively (Kwon and Weissman, 1989; Schwarz et al., 1997). The human CD137 gene consists of eight exons and seven introns and encodes for a 255 aa protein which can exist as either a 30 kD monomer or 55 kD dimer (Kwon et al., 1994).

Expression of the functional glycoprotein is observed on the surface several immune cells including activated $CD4^+$ and $CD8^+$ T cells, monocytes, DCs, follicular DCs (FDCs), NK cells and neutrophils (Futagawa et al., 2002; Garni-Wagner et al., 1996; Lindstedt et al., 2003; Melero et al., 1998; Pauly et al., 2002; Pollok et al., 1993). Non-immune cells such as inflamed vascular endothelial cells, neurons, astrocytes, chondrocytes and microglia have also been reported to express membrane-bound CD137 (Curto et al., 2004; Drenkard et al., 2007; Olofsson et al., 2008; Reali et al., 2003; von Kempis et al., 1997). Furthermore, heightened CD137 expression has also been co-related with several neoplastic pathologies such as in osteosarcoma and Hodgkin's lymphoma (Ho et al., 2013; Lisignoli et al., 1998).

1.4.1.1. Biological activities of CD137

The bulk knowledge pertaining to the functions of CD137 has been deciphered from studies performed in T cells. Expression of CD137 on T cells is strictly activation dependent and is not found on resting T cells (DeBenedette et al., 1995; Wilcox et al., 2002). Upon cross-linkage of CD137 by either its natural ligand or by agonist anti-CD137 antibody, the activity of T cells, especially the $CD8^+$ population, is highly potentiated. Signalling through

CD137 induces T-cell proliferation and production of pro-inflammatory cytokines (Alderson et al., 1994; DeBenedette et al., 1995; Pollok et al., 1993). CD137 acts independently of the CD28-B7 co-stimulatory system as the latter functions to initiate T-cell activation while the former drives T-cell maintenance and viability via inhibition of activation-induced cell death (AICD) and upregulation of anti-apoptotic protein Bcl-XL (Laderach et al., 2002; Starck et al., 2005).

Due to its potent T-cell co-stimulatory potential, *in vivo* activation of T cells using agonist anti-CD137 antibody was proposed to be able to elicit a strong anti-tumour T-cell reaction. Indeed, the antibody treatment was able to supplement the anti-tumour T-cell response to an extent whereby established carcinoma and melanoma were rejected in animal models (Melero et al., 1998). Such was the potency that humanised agonist antibodies are currently being developed for cancer immunotherapy.

Besides T cells, DCs also express surface CD137. Studies by Futugawa et al. and Wilcox et al. showed that triggering CD137 lead to the upregulation of inflammatory cytokines IL-6 and IL-12 and also increased CD80 and CD86 expression, thus essentially priming these cells (Futagawa et al., 2002; Wilcox et al., 2002). Not only does triggering CD137 on DCs lead to a more potent immune response, but its mere presence on splenic DCs is essential for their survival. DCs from CD137 knockout mice were virtually devoid of anti-apoptotic proteins Bcl-2 and Bcl-XL thus making them prone to apoptosis (Choi et al., 2009). Non-immune cells such as epithelial and endothelial cells also expresses CD137 and its ligation boosts cytokine and chemokine secretion that ultimately enhances leucocytes infiltration (Quek et al., 2010; Teijeira et al., 2012).

1.4.2. Biology of CD137 ligand

CD137 interacts with its ligand, CD137L, which is expressed as a type II membrane glycoprotein on the surface of APCs such as monocytes, macrophages, B cells and DCs (Ju et al., 2003; Jung et al., 2004; Kim et al., 2002; Laderach et al., 2003; Lee et al., 2003; Pollok et al., 1994). This member of the TNF superfamily has its gene located on chromosome 19p13.3 and 17 in man and mouse, respectively. The gene contains three exons and two introns and the translated human CD137L polypeptide contains 254 aa with a molecular weight of 34kD (Alderson et al., 1994; Armitage, 1994; Goodwin et al., 1993). As with all members of the TNF superfamily, CD137L protein harbours a TNF domain which is structurally comparable to that of lymphotoxin (LT)-alpha and TNF. In its activated form, CD137L is arranged as homotrimeric or oligimeric aggregates on cellular surface membranes (Rabu et al., 2005; Won et al., 2010).

A peculiar aspect of CD137L is its low degree of sequence homology (15-20%) with other members of the TNF superfamily (Compaan and Hymowitz, 2006). In a study by Won et al., they discovered that human CD137L has structural features that are distinct from other TNF ligands. The trimerised form resembles a three-bladed propeller feature which is unique amongst the TNF superfamily (Won et al., 2010). Additionally, CD137L is able to physically associate with several TLRs including TLR4 to maintain long-term LPS-induced TNF release in murine macrophages, and this is independent of CD137 receptor binding (Kang et al., 2007; Ma et al., 2013). Similarly, CD137L cooperates with TNFR1 to mediate signalling (Moh et al., 2013).

1.4.3. Bidirectional signalling in CD137-CD137L system

An interesting aspect of CD137L biology is its ability to not only interact and activate its receptor, but also signal via its cytoplasmic domain (figure 2). Although rare, this phenomenon of bidirectional signalling has been previously observed in other members of the TNF receptor-ligand superfamily such as TNF, CD40 ligand, CD95 ligand, OX40 ligand and CD30 ligand. Downstream signal transduction of the ligand is known as reverse signalling and an advantage of such two-way signalling is that it allows for efficient communication and cross-talk between two sets of cells. And since the members of TNF-TNFR superfamily are predominantly found in immune cells, this possibly translates to more effective fine-tuning of the immune response (Eissner et al., 2004). Indeed, CD137-CD137L bidirectional signalling cascade occurs during T-cell activation and due to the temporal-dependent increase in their expression, CD137 receptor-ligand system likely provides late-acting signals that allow for the survival of activated T cells. This in turn provides an overall sustained immune response (Croft, 2003).

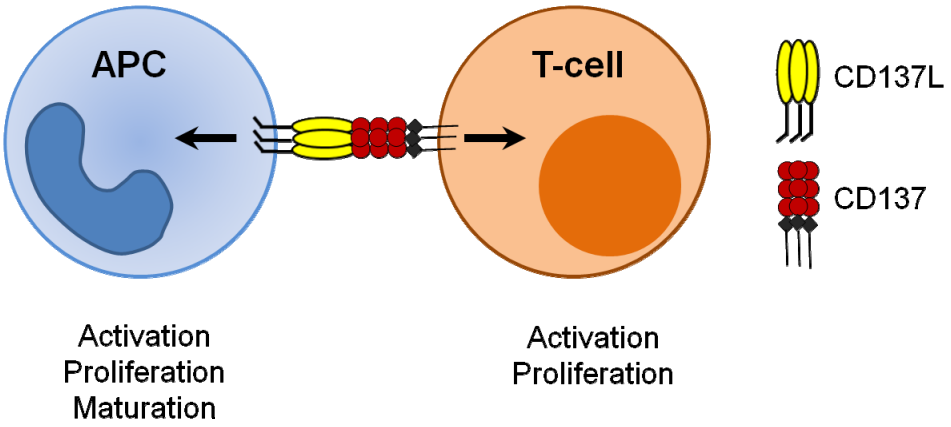


Figure 2. Bidirectional signal transduction and reverse signalling in the CD137 receptor-ligand system. Crosslinking of CD137 and CD137 ligand initiates multiple effects on immune cells.

1.4.4. Reverse signalling

As CD137 is found on activated T cells, it comes as no surprise that CD137L is mainly expressed on APCs such as monocytes and DCs. Besides, CD137L is also expressed on hematopoietic stem and progenitor cells (HSPCs) and reverse signalling in these cells has been shown to greatly impact myelopoiesis, details of which are beyond the scope of this thesis (Tang et al., 2013). In this section, we will discuss the current views of CD137L reverse signalling in DCs and monocytes.

1.4.4.1. Reverse signalling in dendritic cells

DCs are the most potent professional APCs with the capacity to stimulate both memory and naive T cells (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). Upon activation, T cells express high levels of surface CD137 within 24 h which interacts with its ligand on DCs initiating a bidirectional signalling cascade. (Alderson et al., 1994; Hurtado et al., 1995; Pollok et al., 1993).

CD137L is expressed at low levels on human moDCs, $CD34^+$ hematopoietic progenitor cell (HPC)-derived DCs and also on murine DCs. Its expression is strongly enhanced in the presence of inflammatory stimuli such as LPS, double-stranded viral RNA, CD40L and also soluble factors such as TNF and IL-1 (Diehl et al., 2002; Laderach et al., 2003; Lee et al., 2003). The fact that the expression of both CD137 on T cells and CD137 ligand on DCs is enhanced upon initiation of an adaptive immune response indicates the importance of this receptor-ligand interaction for the development of T-cell mediated immunity. Studies have shown that reverse CD137L signalling in DCs, elicited by recombinant CD137 protein, induces TNF secretion which in

turn functions in an autocrine manner to mature DCs as evidenced by the increase in CD83, CD86 and HLA-DR expression (Lippert et al., 2008). Chemokine receptors such as CXCL4 and CCR7, which coordinate DC migration, are also upregulated upon reverse CD137L signalling (Lippert et al., 2008; Van Gool et al., 1996). More importantly, these CD137L-matured DCs are functionally more potent and are able to induce a stronger Th1 response in autologous T cells than classical DCs as shown by high IFN-γ and IL-12p70 secretion (Lippert et al., 2008).

1.4.4.2. Reverse signalling in monocytes

Cross-linking of CD137L on human peripheral monocytes leads to activation as observed by enhanced adherence, morphological changes and production of pro-inflammatory cytokines such as TNF, IL-6 and CXCL8 (Langstein et al., 2000; Langstein et al., 1998; Sollner et al., 2007). Concurrently, secretion of anti-inflammatory IL-10 is downregulated (Langstein et al., 1998). Further, the growth factor M-CSF (CSF-1) is induced by CD137L signalling and autocrine intake of this cytokine is essential for prolonging survival and proliferation of monocytes (Langstein et al., 1999; Langstein and Schwarz, 1999).

Additionally, CD137L activation on monocytes seems to have a role in promoting extravasation. In a pro-inflammatory environment, endothelial cells increase expression of CD137 and interact with circulating monocytes. Quek et al. demonstrated that CD137 on activated endothelial cells enhances intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen (LFA-1) mediated adhesion of monocytes and therefore supports a role of CD137-CD137L in the recruitment of monocytes into inflammatory tissues (Quek et al., 2010).

More recently, our laboratory demonstrated that CD137L signalling can influence the differentiation of human monocytes to become cells with DC characteristics including enhanced CD83 and CD86 expression (Kwajah and Schwarz, 2010). At the same time, these findings were also corroborated by another independent group (Ju et al., 2009). Monocytes treated with recombinant CD137 protein or with agonistic anti-CD137L antibody plus IL-4 for a minimum of four days gained the capacity to stimulate allogeneic T cells, preferentially towards an inflammatory phenotype as apparent by the presence of IFN-γ, in both $CD4^+$ and $CD8^+$ T cells, and IL-17 together with the reduction of IL-4 (Ju et al., 2009; Kwajah et al., 2011; Kwajah and Schwarz, 2010). Moreover, these CD137L-stimulated DCs (CD137L-DCs) inhibit Treg development as the expression of the Treg-specific transcription factor, Foxp3, is reduced (Kwajah and Schwarz, 2010). Even more intriguing was the heightened effectiveness of these CD137L-DCs when compared to classical DC (generated by GM-CSF plus IL-4). Allogeneic T cells co-cultured with CD137L-DCs were more effective in a MLR compared to classical DCs matured by LPS plus IFN-γ or by TNF. Altogether, CD137L-DCs are able to mount a potent pro-inflammatory response suggesting their potential use for immunotherapy.

Although CD137L-DCs promote adaptive inflammatory responses, this activity is evident only after four days of treatment with CD137-Fc protein. During the initial stages, CD137L-signaling into monocytes actually induces T-cell apoptosis instead of promoting T-cell proliferation (Kwajah et al., 2011; Michel et al., 1999; Schwarz et al., 1996). A recent study showed that the interaction of CD137-Fc-treated monocytes with T cells initiates ROS production in the latter leading to their demise by apoptosis (Kwajah et al., 2011). Though seemingly counterproductive for an immune response, such a

mechanism has been described as infection-induced T-cell attrition where there is a massive clearance of non-specific T cells early in an anti-pathogen response thereby facilitating the later expansion of antigen-specific T cells, and an effective anti-pathogen immune response (Bahl et al., 2006; Jiang et al., 2003; McNally et al., 2001).

1.4.4.3. Differential species response towards CD137L reverse signalling

To further characterise CD137L-DCs for immunotherapy, a study using the murine system as a model was undertaken (Tang et al., 2011). Similar to the response by human monocytes, CD137-Fc treated $CD11b^{+}Ly6G^{-}$ monocytes displayed enhanced adhesion, morphological changes, increased proliferation and CD80 and CD86 expression. However, the upregulation of F4/80 and CD11c expression prompted the idea that these cells were both macrophage-like and DC-like. Several differences such as increased IL-10 secretion and enhanced phagocytosis meant that perhaps reverse signalling induces different responses in different species. Critically, CD137-Fc treatment of murine monocytes was not able to stimulate T cells. Also, the inability of CD137L reverse signalling to activate immature DCs is strong evidence that there is a species difference in the effects of CD137L signalling between human and murine monocytes (Tang et al., 2011). This discrepancy in the functional biology of CD137L between mouse and man prevents the application of the murine system as an avenue for *in vivo* studies.

1.5. Research scope and objectives

The recent development of CD137L-DCs, generated by inducing CD137L signalling into human peripheral monocytes, is of clinical relevance as they can potentially be use for more efficient DC-based immunotherapy protocols. These novel DCs are better equipped to activate allogeneic T cells as evidenced by the potent induction of T-cell proliferation, high IFN-γ production in $CD4^+$ T cells and high perforin in $CD8^+$ CTLs (Ju et al., 2009; Kwajah and Schwarz, 2010). However, nothing is known about their efficiency to induce autologous, antigen-specific immune response. Further characterisation of these CD137L-DCs such as determining their ability to initiate antigen-specific T cells should be looked upon. These data would provide essential information for the use of these CD137L-DCs for future immunotherapeutics. Therefore, this project aims to investigate the following;

i. *Maturation factors required to further activate CD137L-DCs*

Activation by LPS plus IFN-γ was shown to be unable to increase the T-cell activating capacity of CD137L-DCs. However, certain aspects of maturation such as an increase in CD86 and HLA-DR expression were observed. Thus, this suggested that CD137L-DCs can be further matured and activated by an optimised maturation cocktail.

ii. *The ability of CD137L-DCs to stimulate autologous T cells in an antigen-specific manner*

CD137L-DCs are potent stimulators of allogeneic T cells. This feature has not been tested in an autologous, antigen-specific setting, and it is therefore critical that it is characterised in order to identify the potential use of CD137L-DCs in a DC-based immunotherapy.

iii. *Effects of CD137L-DC-stimulated antigen-specific T cells towards tumour cells*

Upon re-stimulation by CD137L-DCs, the T-cell function and their potency has to be resolved. Hence, it is of interest to determine if these alternatively stimulated T cells are able to potently induce the demise of tumour cells.

iv. *Transcriptional profiling of CD137L-DCs*

To better understand the key elements that make CD137L-DCs potent APCs, transcriptional profiling can be undertaken. This may shed light onto the specific genes and proteins that are differentially regulated upon CD137L reverse signalling in monocytes and may be essential in the functional biology of CD137L-DCs.

Essentially, it is anticipated that these findings will strengthen the justification for the use of CD137L-DCs for cancer immunotherapy.

CHAPTER 2: MATERIALS AND METHODS

2.1. Recombinant proteins, antibodies and reagents

Recombinant human CD137-Fc fusion protein was purchased from R&D Systems. It consist of an extracellular domain of the human CD137 protein (Leu24-Gln186) fused with a human Fc fragment from human IgG1 (Pro100-Lys186) with a linker sequence of DIEGRMD linking the two portions. Human IgG1 Fc fragment was purchased from Millipore. GM-CSF, M-CSF, IL-4, TNF, IL-1β, IL-6, IL-7, IL-15 and IFN-γ were purchased from Peprotech (NJ, USA) while IL-2 was from R&D Systems. Lipopolysaccharide (LPS) derived from *E.coli* of serotype 0111:B4 and prostaglandin E2 (PGE2) were obtained from Sigma-Aldrich. Resiquimod (R848), poly (I:C) and recombinant flagellin were purchased from Invivogen. Anti-human antibodies procured were applied to either flow cytometric analysis or neutralisation studies. The full list of antibodies employed for the project is shown in table 2.

Table 2. List of antibodies

Human antigen	Conjugate	Clone	Vendor
ALCAM (CD166)	Unconjugated	105901	R&D Systems
	PE	3A6	eBioscience
CCR7	APC	3D12	eBioscience
CD137	PE	4B4-1	BD Pharmigen
CD137L	Unconjugated	41B436	AdipoGen
CD14	PerCP-Cy5.5	61D3	eBioscience
CD154	eFluor® 450	24-31	eBioscience
	PerCP-eFluor® 710	24-31	eBioscience
CD20	APC	2H7	eBioscience
CD3	FITC	SK7	eBioscience
	APC	SK7	eBioscience
CD4	eFluor® 605NC	OKT4	eBioscience
	Alexa Fluor® 700	OKT4	eBioscience
CD70	PE	113-16	BioLegend
CD8	APC	SK-1	eBioscience
CD80	PE	2D10.4	EBioscience
CD83	APC	HB15E	EBioscience
CD86	PE	IT2.2	eBioscience
CLEC5A	APC	283834	R&D Systems
CXCR4	PE	12G5	eBioscience
HLA-A2	PE	BB7.2	BD Pharmigen

HLA-ABC	FITC	W6/32	eBioscience
HLA-DR	eFluor® 450	L243	eBioscience
ICAM-1 (CD54)	PE	HA58	eBioscience
IFN-γ	PE	4S.B3	eBioscience
	eFluor® 450	4S.B3	eBioscience
Mouse IgG1κ	Unconjugated	MOP-C21	Sigma-Aldrich
OX40L (CD252)	PE	11C3.1	BioLegend
PDPN	APC	NZ-1.3	eBioscience
TNF	FITC	MAb11	eBioscience

Calcium ionophore A23187 and phorbol myristate acetate (PMA) were obtained from Sigma-Aldrich. CytoStim (human), an antibody-based reagent that functions to restimulate T cells in the presence of APCs, was obtained from Miltenyi Biotec. Brefeldin A was purchased from eBioscience.

2.2. Protein and peptide antigens

CMV pp65 recombinant protein and tetanus toxoid (*Clostridium tetani*) were procured from Miltenyi Biotec and Calbiochem (Merck) respectively.

PepTivator® CMV pp65, EBV LMP2A and melanoma MelanA/MART-1 antigen peptide pools were purchased from Miltenyi Biotec. Each peptide pool is composed of 15-mer sequence with 11 amino-acid overlap, covering the complete sequence of the human pp65 protein of CMV strain AD169 (Swiss-Prot Acc. no. P06725), LMP2A protein of EBV strain B95-8 (Swiss-Prot Acc. no. P13285) and human MelanA/MART-1 protein (Swiss-Prot Acc. no. Q16655), respectively. Immunodominant pp65 NLVPMVATV (NLV) peptide was a kind gift from Dr. Paul MacAry (National University of Singapore).

2.3. Isolation of primary cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from either buffy coat or fresh blood of healthy donors and were prepared by Ficoll-Paque (GE Healthcare) density gradient centrifugation. PBMCs were washed with PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA), (PBS 2 mM EDTA). When required, red blood cells were lysed by a lysis buffer. PBMCs were washed at least twice more with PBS 2 mM EDTA. PBMCs were either used whole or cellular subsets were subsequently isolated.

Monocytes were isolated from prepared PBMCs by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec) according to manufacturer's instructions. The kit consists of a cocktail of biotin-labelled antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A. In brief, whole PBMCs were treated with the antibody cocktail and FcR blocking reagent for 10 min followed by incubation with anti-biotin microbeads for a further 15 min. Treated PBMCs were then passed through a column in a strong magnetic field in which cells bound to the antibodies were retained while desired monocytes were collected as column flow-through. Isolated monocytes were >95 % pure based on antigenic phenotyping by CD14 staining.

Isolation of pan T cells was performed using anti-CD3 microbeads (Miltenyi Biotec). After 15 min of incubation with microbeads, PBMCs were passed through a column in a strong magnetic field. Flow-through was discarded while desired cells were retained in the column and were flushed out after removal from the magnetic field. Isolated pan T cells were >98 % pure based on antigenic phenotyping by CD3 staining.

2.4. Generation of DCs and macrophages, and maintenance of EBV-transformed B lymphoblasts

To generate CD137L-DCs, freshly isolated monocytes were seeded onto 6-well polystyrene plates (Becton Dickinson) pre-coated with 10 μg/ml of CD137-Fc protein, in RPMI-1640 supplemented with 10 % FBS, 50 μg/ml streptomycin and 50 IU/ml penicillin (R10 PS media) for 7-8 days. Final density was 10^6/ml. When further maturation with various maturation cocktails was performed, this was done during the final 18 h of incubation.

Classical DCs were generated by culturing monocytes at a density of 10^6/ml in R10 PS media in the presence of GM-CSF (80 ng/ml) and IL-4 (100 ng/ml) for a total of 7-8 days on 12-well polystyrene plates. Fresh media was added on day 3. For the last 18 h, cells were matured with either LPS (1 μg/ml) plus IFN-γ (50 μg/ml) or with TNF (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml) and PGE2 (1 μg/ml).

Macrophages were generated by culturing monocytes in R10 PS media in the presence of M-CSF (100 ng/ml) for a total of 7-8 days. Fresh media was added on day 3.

HLA-$A2^+$ EBV-transformed B lymphoblasts (clone CM371) were a gift from Dr. Laura Rivino (National University of Singapore) and were maintained in R10 PS media.

2.5. Antigen-recall assay

To determine responders to several different recall antigens, isolated donor PBMCs were resuspended in RPMI supplemented with 2 % human Ab serum (Sigma-Aldrich), 50 μg/ml streptomycin and 50 IU/ml penicillin (R2Ab PS media) at 10^7/ml and seeded into 96-well round-bottom plates at 100 μl well.

Various PepTivator® peptide pools (pp65, LMP2A or MelanA/MART-1) were added at 1 μg/ml per peptide and incubated at 37ºC for 18 h. Unpulsed and CytoStim conditions acted as negative and positive controls, respectively. When intracellular staining was desired, Brefeldin A was added 2 h after initiation of restimulation with peptide pools.

To study the potency of CD137L-DCs to restimulate antigen-specific T cells, compatible donors were recruited. Classical and CD137L-DCs were generated, matured with TNF (50 ng/ml) or LPS (1 μg/ml) for 16-18 h and then pulsed with pp65 NLV peptide (1 μg/ml), pp65 peptide pool (1 μg/ml per peptide) or LMP2A peptide pool (1 μg/ml per peptide) for 1-2 h at 37ºC in R2Ab PS media. Unpulsed and CytoStim conditions acted as negative and positive controls, respectively. In cases where whole protein tetanus toxoid was used as antigen of interest, pulsing of antigen (1 or 5 μg/ml) and maturation were performed simultaneously for 16-18 h. After washing with PBS, pulsed DCs were co-cultured with autologous pan T cells at a ratio of 1:10. Co-cultures were incubated at 37ºC for either 18 h for flow cytometric and ELISA analyses or 5 days for ^{3}H-thymidine proliferation analysis.

2.6. Maturation of CD137L-DCs and classical DCs

Monocytes were seeded onto CD137-Fc coated plates, and on day 6 various cytokine cocktails (refer to tables 4, 5 or 6) were added into the culture for an additional 18 h to induce maturation. Cells were then analysed for expression of CD14, CD80, CD83, CD86, CCR7, CXCR4, HLA-ABC, and HLA-DR. Supernatants were harvested and tested for IL-12p40, IL-12p70 and IL-23 secretions by ELISA. Additionally, their ability to induce T-cell activation as

measured by proliferation of allogeneic T cells and IFN-γ production was also tested.

For autologous, antigen-specific experiments, CD137L-DCs were matured using the optimized cocktail of R848 (1 μg/ml) plus IFN-γ (50 ng/ml) while classical DCs were matured by TNF (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml) and PGE2 (1 μg/ml) for 18 h.

For transcriptional profiling assays, classical DCs were matured by LPS (1 μg/ml) plus IFN-γ (50 ng/ml) for 18 h.

2.7. Dissociation assay upon DC maturation

Monocytes were seeded onto CD137-Fc coated plates, and on day 6 various cytokine cocktails (refer to table 4) were added into the culture for an additional 18 h to induce maturation. Culture supernatants were decanted, cells gently washed twice with PBS and subsequently treated with 1 × trypsin-EDTA. Culture plates were placed on a bidirectional rotator with gentle agitation to enhance cell dissociation. The time required (at a resolution of 30 seconds) for complete dissociation of DCs from the culture plate was recorded. Each condition was assayed with a minimum of triplicates.

2.8. Allogeneic mixed-lymphocyte reaction

Allogeneic mixed-lymphocyte reaction (MLR) was performed to determine the ability of DCs to induce T-cell proliferation and activation.

Day 7-8 DCs were co-cultured with allogeneic pan T cells at a ratio of 1:10 (10^4 DCs and 10^5 T cells). T cells were either used unstained or pre-treated with CFSE. Cultures were incubated at 37ºC in R10 PS media for 5 days and cell proliferation was determined via CFSE dilution by flow cytometry.

Supernatants from co-cultures were collected and cytokine production was determined by ELISA.

2.9. Generation of pp65 T-cell lines

PBMCs were suspended at 10^6 cells/ml in AIM V media (Invitrogen) supplemented with 2 % human Ab serum (Sigma-Aldrich), streptomycin sulphate at 50 μg/ml, and gentamicin sulphate at 10 μg/ml (AIMV 2Ab media). They were then stimulated with PepTivator pp65 spanning peptide pool (0.1 μg/ml per peptide; Miltenyi Biotec) for 9-10 days supplemented with IL-2 (10 U/ml) added after 24 h to generate a peptide-derived pp65 T-cell line. To generate protein-derived pp65 T-cell line, 5 μg/ml of pp65 recombinant protein (Miltenyi Biotec) was added instead. On day 9-10, >90 % and >95 % of cells were $CD3^+$ in the protein-derived and peptide-derived T-cell lines, respectively. Cells were washed at least once with PBS and resuspended in fresh AIMV 2Ab media before they were used for further experiments.

2.10. Restimulation of pp65 T-cell lines

2.10.1. Restimulation of peptide-derived pp65 T-cell line

After overnight maturation, CD137L-DCs or classical DCs were harvested and resuspended in AIM V media. Cells were pulsed with the pp65 peptide pool at various concentrations (0.01, 0.1 and 1 μg/ml per peptide) for 2 h at 37ºC. As a negative control, the pp65 peptide pool was not added into the culture. Cells were washed once and resuspended in AIMV 2Ab media. Pulsed DCs were co-cultured with autologous peptide-derived pp65 T-cell line at a ratio of 1:10 and was incubated for a total of 18 h. Brefeldin A at a

working concentration of 2 μg/ml was added after the initial 2 h of co-culture. Samples were surface stained for CD3, CD4 and CD8 while intracellular staining was performed to detect IFN-γ, TNF and CD154 before being subjected to flow cytometric analysis.

For the 5 day co-culture experiment, untreated/mature DCs were pulsed with the pp65 peptide pool at a concentration of 0.1 μg/ml per peptide and were left in culture throughout. The autologous peptide-derived pp65 T-cell line was co-cultured with DCs at a ratio of 10:1 before supernatants were obtained after 5 days. Supernatants were kept at -20ºC until analysed by ELISA for IFN-γ, IL-10, IL-13, IL-17 and TNF.

2.10.2. Restimulation of protein-derived pp65 T-cell line

On day 6-7 of CD137L-DC or classical DC generation, cells were harvested, resuspended in AIMV 2Ab media and seeded onto culture plates. DCs were then activated with their respective optimised maturation cocktail for 18 h. Concurrently, DCs were pulsed with 0.385, 3.85 or 38.5 μg/ml (0.1, 1 and 10 ×, respectively) of pp65 recombinant protein. As a negative control, the pp65 recombinant protein was not added into the culture. After maturation and antigen-pulsing, cells were washed once and resuspended in AIMV 2Ab media. Pulsed DCs were co-cultured with autologous protein-derived pp65 T-cell line at a ratio of 1:10 and were incubated for 18 h. Samples were surface stained for CD3, CD4, CD8 and CD137 before being subjected to flow cytometric analysis.

2.11. Cytotoxic assay

For the pp65-specific cytotoxic assay, the pp65 T-cell line was restimulated with the pp65 peptide pool (0.1 μg/ml per peptide) pulsed DCs at a ratio of

10:1 for 5 days. Cells were harvested, resuspended in AIM V media supplemented with 2 % human Ab serum, 50 μg/ml streptomycin sulphate, 10 μg/ml gentamicin sulphate, 2 mM probenecid and 50 μM β-mercaptoethanol (AIMV 2Ab PM media). T cells were surface stained for CD3 (> 95 %).

Target HLA-matched EBV-transformed B lymphoblasts (clone CM371) were loaded with DELFIA® BATDA Reagent and a cytotoxicity assays was performed according to manufacturer's instruction (DELFIA® EuTDA Cytotoxicity Reagents; Perkin Elmer). This assay has been described to be more sensitive than the conventional ^{51}Cr release assay(von Zons et al., 1997). In brief, target cells were harvested and washed once in R10 PS before they were resuspended in R10 PS media supplemented with 2 mM probenecid and 50 μM β-mercaptoethanol (R10 PS PM media) at a density of 10^6 cells/ml. 5 μl of fluorescence enhancing ligand (DELFIA® BATDA Reagent) was added to every 4×10^6 target cells followed by an incubation for 20 min at 37ºC. Labelled cells were washed for 3-4 times in R10 PS PM media and were then resuspended in AIMV 2Ab PM media. Loaded target cells were then pulsed with pp65 peptide pool (1 μg/ml per peptide) for 1 h at 37ºC. Following which, they were washed and seeded into 'V'-bottom 96-well plates at 10^4 cells per well.

To initiate the killing assay proper, pp65-specific effector T cells were co-cultured with target cells at E:T ratios of 5:1, 10:1 and 20:1 for 3 h at 37ºC. The Europium solution was added to the harvested supernatants for 15 min before the fluorescence was measured using a time-resolved fluorometer (VICTOR^3 multilabel reader: PerkinElmer).

2.12. Microarray processing and analysis

2.12.1. RNA extraction and cRNA synthesis[1]

Total RNA was extracted following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol: Invitrogen) followed by a Qiagen RNeasy® clean-up procedure. RNA purity was assessed by spectrophotometry. Total RNA integrity was assessed by Agilent Bioanalyzer and only high quality RNAs, with an RNA Integrity Number (RIN) greater than 7, were considered for microarray analysis. Biotinylated cRNA was prepared according to the Epicentre TargetAmp™ Nano-g™ Biotin-aRNA Labelling kit for Illumina system. Very strict quality control measures were applied during the target preparation procedures. cRNA was analysed by Agilent Bioanalyzer. [1] This procedure was performed me and Ms. Josephine Lum from A*STAR (Singapore).

2.12.2. Standard array hybridisation[2]

Illumina Human HT12v4 Beadchips for whole-genome gene expression were used as the standard array. Labelled cRNAs were hybridized to the standard arrays for 16 hours at 58ºC; the arrays were then washed and stained base on Illumina Wash Protocol and then scanned using BeadArray Scanner 500GX at BSF Microarray Facility. [2] This procedure was performed by Ms. Josephine Lum from A*STAR (Singapore).

2.12.3. Expression data analysis and selection of differentially expressed genes[3]

The expression data were background subtracted in Genome Studio and further processed with quantile normalisation and log2 transformation.

Differential expression analysis was done with the linear model for microarray data (limma) bioconductor package and the differentially expressed genes were selected with Benjamini-Hochberg multiple testing correction adjusted *p*-value < 0.05. The cluster dendrogram and cMAP analysis were generated using a freeware program called R. The CD137L-DC signature probes were selected by obtaining the gene set that is found in the intersection of differentially expressed genes of CD137-Fc treatment vs monocytes and differentially expressed genes of CD137-Fc vs Fc (figure 34). This allowed the acquisition of differentially expressed genes which are contributed solely by CD137L reverse signalling into monocytes. A total of 974 probes, corresponding to 829 genes, were identified as CD137L-DC differentially expressed genes. [3]This procedure was performed by me and Dr. Kaibo Duan from A*STAR (Singapore).

2.12.4. DAVID analysis

To determine the unique functional characteristics of CD137L-DCs against each of the subtypes, differentially expressed genes with >2 fold change in each group were subjected to Gene Ontology (GO) biologic process using Database for Annotation, Visualization and Integrated Discovery (DAVID) tools (Huang da et al., 2009). Categories with significant enrichment (*p* value < 0.05) were acquired and similar categories were grouped together. Refer to appendix II to view the complete list of genes used for DAVID analysis.

2.13. Neutralisation assay

Day 7 CD137L-DCs were harvested and resuspended in fresh R10 PS media at 2×10^5 cells/ml. 50 μl of DC suspension was added into each well of a 96-

well round bottom plate. Antagonistic anti-ALCAM Ab (R&D Systems) or control mouse IgG1κ isotype (MOPC-21) was added into appropriate wells at 10 μg/ml. Plates were incubated for 1 h in 37ºC before allogeneic T cells was added to give a final DC:T-cell ratio of 1:10 in total volume of 100 μl/well. Culture plates were incubated for a total of 4 days and ^{3}H-thymidine was added for the final 18 h of culture.

To generate supernatants for ELISA purposes, co-culture conditions were scaled-up to a total volume of 400 μl/well in 48-well plates. Cultures were incubated for 7 days before supernatants were harvested and kept in -20ºC freezer until analysis.

2.14. Attachment assay

Day 7 generated CD137L-DCs and classical DCs were incubated with 10 mM EDTA/non-enzymatic cell dissociation medium for 20 min followed by cell scraping for a complete harvest of cells. Cells were resuspended in R10 PS media and seeded into individual wells of a 48-well plate at 10^{5} cells/well. The culture plate was centrifuged for 5 min at 1,500 rpm to allow cells to descend onto the bottom before incubation at 37ºC.

At the time-points of 10, 45 and 90 min, supernatants containing suspended cells were gently collected and washed once with PBS without flushing. Supernatants and washing PBS were pooled and labelled as ‘unattached cells’. DCs that had adhered to the wells were harvested by treatment with trypsin-EDTA for 10 min followed by flushing with PBS to ensure complete dissociation of cells. These collected cells were labelled as ‘attached cells’. Absolute numbers of cells in each condition were counted using

CountBright™ absolute counting beads. Propidium iodide (PI) was added for dead cell exclusion.

2.15. Detachment assay

Day 7 generated CD137L-DCs and classical DCs were incubated with 10 mM EDTA/non-enzymatic cell dissociation medium for 20 min followed by cell scraping for a complete harvest of cells. Cells were resuspended in R10 PS media and seeded into individual wells of a 48-well plate at 10^5 cells/well. The culture plate was centrifuged for 5 min at 1,500 rpm before incubation at 37ºC for 18-20 h to allow for a complete attachment of DCs.

Supernatants were harvested and wells were gently washed once with PBS. Cells were detached from the substratum by treatment with typsin-EDTA for 5, 10 or 15 min at 37ºC. At the indicated time-points, cells were harvested followed a single gentle wash with PBS. Trypsin-EDTA and washing PBS were pooled and labelled as 'detached cells'. The absolute number of cells in each condition was counted using CountBright™ absolute counting beads. PI was added for dead cell exclusion.

2.16. Cell counting

Cell counting was performed either by using a haemocytometer or by flow cytometry with counting beads.

2.16.1. Manual counting using a haemocytometer

Cells were harvested upon incubation with trypsin-EDTA or 10 mM EDTA/non-enzymatic cell dissociation medium (Sigma-Aldrich). Sterile cell

scrapers were used to facilitate complete detachment of cells from culture plates. Cells were then washed and resuspended in PBS or media following which they were mixed at 1:1 ratio with 0.4 % trypan blue solution (Sigma-Aldrich T8154). Cells were counted on a haemocytometer (Neubauer) under a bright field microscope.

2.16.2. Cell counting beads assay

Harvested cells were resuspended in a known volume of staining buffer in flow cytometry tubes. 5-10 μl of CountBright™ absolute counting beads (Invitrogen) were added into each tube before the sample was ran on the flow cytometer. The absolute cell number in each sample was calculated using the following formula.

(A/B) × (C/D) = concentration of sample as cells/μl.

Where A = number of cell events, B = number of bead events, C = assigned bead count of the lot (beads/5 or 10 μl), D = volume of sample in μl.

2.17. Quantification of cell proliferation

Two methods were used to determine cell proliferation; the tritium (^{3}H)-thymidine incorporation assay and the CFSE dilution assay.

2.17.1. ^{3}H-thymidine incorporation assay

During the final 18 h of incubation, cells were pulsed with 0.5 μCi of ^{3}H-thymidine (PerkinElmer) after which the culture plate was kept in a -20ºC freezer to disrupt cellular integrity. The culture plate was then thawed at room temperature and the content was transferred onto a Packard Unifilter Plate

using a MicroMate 196 Cell Harvester and counted using TopCount (PerkinElmer) liquid scintillation and luminescence counter. Each condition was assayed at least in triplicates.

2.17.2. CFSE dilution assay

For CFSE-based assays, CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) was used. T cells were harvested, washed and were thoroughly resuspended in sterile PBS supplemented with 0.1 % BSA (CFSE buffer) to a density of 10^6 cells/ml. The CFSE stock solution was made freshly prior to every experiment by dissolving lyophilised CFSE powder into 18 μl of DMSO. 8 μM of stock CFSE dye was added into the T-cell suspension and the mixture was incubated for 10 min at 37ºC water bath. Staining was then quenched by the addition of 5 volumes of ice-cold R10 PS media to the cells following which they were incubated for a further 5 min in ice. Cells were washed, resuspended in media and used for further experiments.

2.18. Flow cytometry

In order to determine surface marker expression, cells were harvested and had their Fc receptors blocked by incubating cells with FcR blocking reagent (Miltenyi Biotec) for 10 min at 4ºC. Cells were then stained with antibodies in PBS containing 0.5 % BSA and 0.1 % sodium azide (staining buffer) for 45 min at 4ºC in the dark. Cells were washed twice and resuspended in 400 μl of staining buffer. Unstained samples or samples stained with the indicated isotype antibody were used as controls.

For intracellular cytokine staining, cells were cultured in the presence of 2 μg/ml brefeldin A (eBioscience) overnight and stained with anti-CD8 or anti-

CD4 antibody. This was followed by cell fixation and permeabilisation using BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences).

Flow cytometry was carried out with CyAn ADP Analyzer (Dako) or BD LSR Fortessa cell analyzer (BD Bioscience). Data were analysed using the Summit or FlowJo data acquisition and analysis software.

2.19. Intracellular cytokine staining

Brefeldin A (2 μg/ml) was added 2 h after the start of co-culture between DCs and T cells to halt protein transport within the cells, thereby preventing secretion of cytokines and proteins. Cells were harvested and blocked for Fc receptors by incubating cells with FcR blocking reagent (Miltenyi Biotec) for 10 min at 4ºC. Upon which, cells were surface stained for CD3, CD4 or CD8 using the respective anti-human antibodies. To stain intracellular cytokines, cells were initially fixed and permeabilised using the BD Cytofix/Cytoperm^{TM} fixation/permeabilisation kit (BD Bioscience) according to manufacturer's protocol. Briefly, cells were fixed and permeabilised using the BD Cytofix/Cytoperm solution for 20 min at 4ºC. Next, cells were washed twice in 1 × BD Perm/Wash solution. After fixation and permeabilisation, cells were stained intracellularly for IFN-γ, TNF or CD154 using the appropriate antibodies in BD Perm/Wash solution.

2.20. ELISA

The concentrations of CXCL8, IFN-γ, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and IL-13 in cell supernatants were determined by human CXCL8, IFN-γ, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-13 ELISA Duoset (R&D Systems), respectively. IL-17 and IL-23 were detected by human IL-17 and IL-23 Ready-

SET-Go!® ELISA kits, respectively (eBioscience). All measurements were performed in triplicates.

ELISAs were performed according to manufacturer's protocol. In brief, samples were incubated for 2 h in wells of Nunc MaxiSorp® flat-bottom 96 well plates pre-coated with the appropriate capture antibody. This was followed by further 1-2 h incubation with appropriate detection antibody, 30 min with streptavidin-HRP and eventually 10-20 min with TMB substrate (eBioscience). In between each incubation step, the wells were thoroughly washed with PBS containing 0.05 % Tween®20. Upon development of the TMB substrate, the reaction was stopped with 2N H_2SO_4 before being analysed using BioRad 680 microplate reader or VICTOR^3 multilabel reader (PerkinElmer).

2.21. Photographs

The morphology of cells was documented using the Zeiss Axiovert 40 inverted microscope (Zeiss, Gottingen, Germany) and Canon PowerShot G6 digital camera.

2.22. Statistical analysis

Statistical significance was determined by a two-tailed unpaired Student's *t*-test unless specified otherwise.

CHAPTER 3: RESULTS

3.1. CD137L is expressed and is functional in primary human monocytes

3.1.1. Expression of CD137L on monocytes

It was previously reported that CD137L is expressed constitutively on human peripheral monocytes and also on promyeloblastic cells lines such as HL-60 (Ju et al., 2003; Laderach et al., 2003). To verify these previous findings, the expression of CD137L on several donor peripheral monocytes was determined by flow cytometry. Donor PBMCs were gated for the monocytic population using forward and side scatter and this was followed by $CD14^+$ expression to confirm the monocyte population. In two representative donors, about 43-48% of $CD14^+$ cells were positive for CD137L surface expression thus confirming the previous reports (figure 3). Such an expression profile is similar to that reported by Ju and colleagues on peripheral monocytes (Ju et al., 2003)

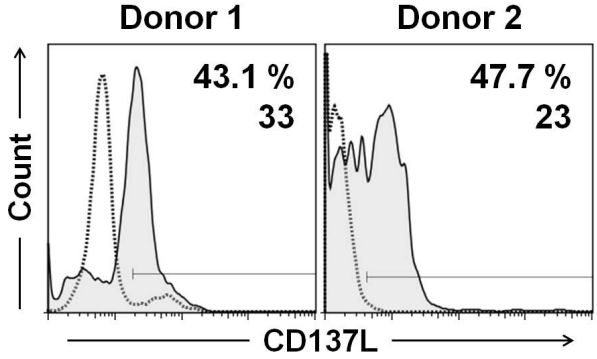


Figure 3. CD137L is expressed on peripheral monocytes. PBMCs isolated from buffy coats of 2 individual healthy blood donors were immunostained for CD137L using the anti-CD137L primary antibody, clone 41B-436, followed by goat anti-mouse-PE conjugate antibody. Percentage of CD137L expressing cells in $CD14^+$ population was determined by flow cytometry. Unshaded and grey histograms represent isotype control and anti-CD137L stained cells, respectively. Values in histogram represent percentages of positive cells and MFI.

3.1.2. CD137L reverse signalling induces pro-inflammatory cytokine secretion in monocytes

Cross-linking CD137L on peripheral monocytes by CD137-Fc protein or agonistic antibodies induces expression of pro-inflammatory cytokines and factors such as M-CSF that are essential for prolonged monocyte survival (Laderach et al., 2003; Langstein et al., 2000; Langstein et al., 1998; Langstein and Schwarz, 1999). This was duly observed in the supernatants of monocytes treated with CD137-Fc proteins for 18 h. Upon stimulation through CD137L, monocytes produced large amounts of TNF, IL-1β, IL-6 and CXCL8 (454 ± 43, 92 ± 14, 421 ± 15 and 22678 ± 315 pg/ml, respectively) and were significantly higher than Fc-treated monocyte controls (figure 4). Similarly, when compared to GM-CSF plus IL-4 or M-CSF treated monocytes, which will skew their differentiation towards classical DCs and macrophages, respectively, CD137L reverse signalling induced an inflammatory condition as demonstrated by the significantly higher production of pro-inflammatory cytokines.

These experiments confirmed that CD137L is indeed expressed on human peripheral monocytes and downstream reverse signalling is functional, leading to an inflammatory status.

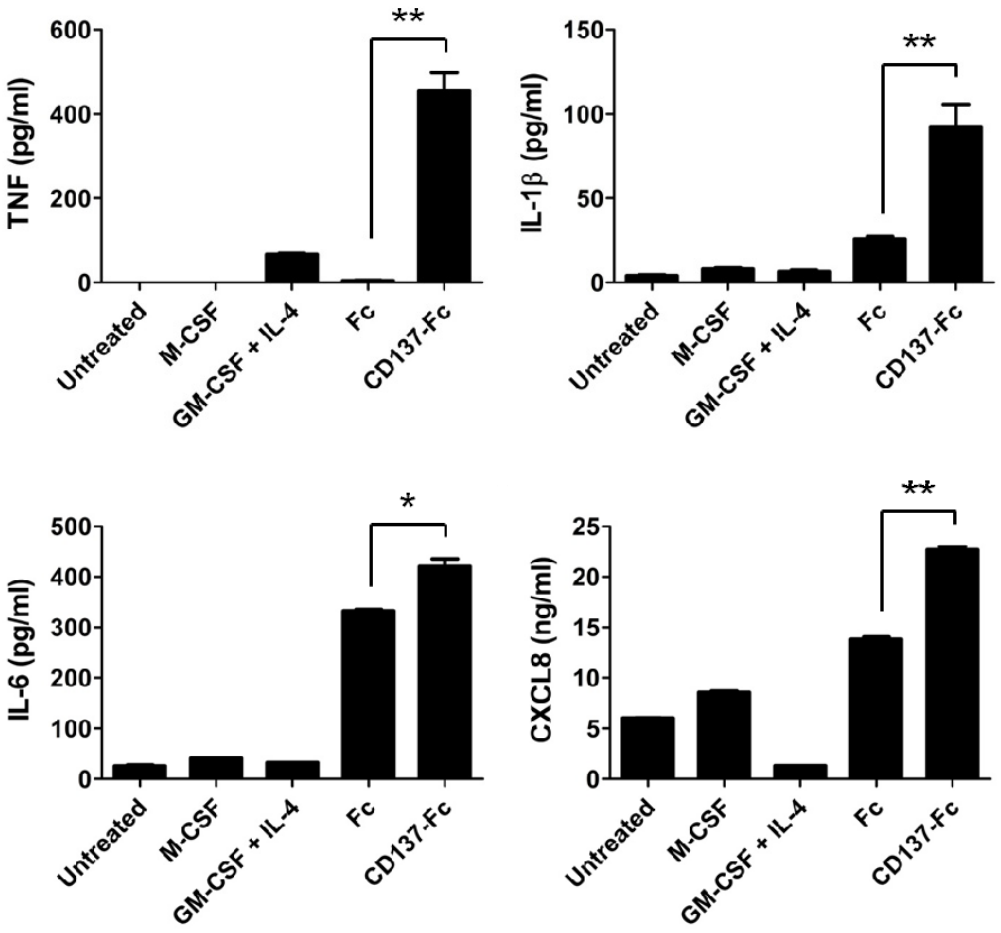


Figure 4. CD137L reverse signalling activates monocytes. Isolated monocytes were cultured untreated (PBS), or with Fc or CD137-Fc treatments. Additionally, monocytes were also cultured in the presence of 100 ng/ml M-CSF or 80 ng/ml GM-CSF + 100 ng/ml IL-4 to generate macrophages and classical DCs, respectively. After 18 h of culture, supernatants were harvested and levels of inflammatory cytokines, TNF, IL-1β, IL-6, and CXCL8 were determined by ELISA. Depicted are means ± standard deviations of triplicate measurements. *p<0.05; **p<0.01 using a two-tailed unpaired Student's t-test.

3.2. Characterising CD137L-DCs for antigen recall capacity

Two recent studies identified a new method for generating human DCs of enhanced potency. Activation of CD137L by crosslinking with CD137-Fc protein or agonistic anti-CD137L antibody was able to activate and differentiate monocytes into inflammatory DCs (Ju et al., 2009; Kwajah and Schwarz, 2010). These CD137L-generated DCs, henceforth referred to as CD137L-DCs, can potently activate T cells leading to a stronger proliferation and higher cytokine release in an allogeneic MLR than classical DCs. Preliminary results also point towards a more potent cytotoxic capacity by these CD137L-DC-activated T cells (personal communication by Dr. Shaqireen Kwajah). Although these earlier studies yielded very promising data they left open whether CD137L-DCs have superior potency in an autologous setting which is critical for their potential use in tumour immunotherapy.

3.2.1. HLA-A2 haplotyping of donors

To study the ability of CD137L-DCs in an autologous condition, donors of a specific haplotype and with the ability to respond to specific antigens have to be initially identified. Individuals with a positive haplotype for HLA-A2 were of interest due to the vast availability of antigens and HLA-matched cell lines which could be potentially used for recall studies and killing assays.

A total of 8 potential donors were recruited and tested for HLA-A2 expression on their PBMCs. Flow cytometry analysis showed that donors 1-4 were HLA-$A2^+$ while donors 5-8 were HLA-$A2^-$ (figure 5). Despite being HLA-$A2^-$, donors 5-8 were also tested for their response to various antigen peptide pools, and this will be described in section 3.2.3.

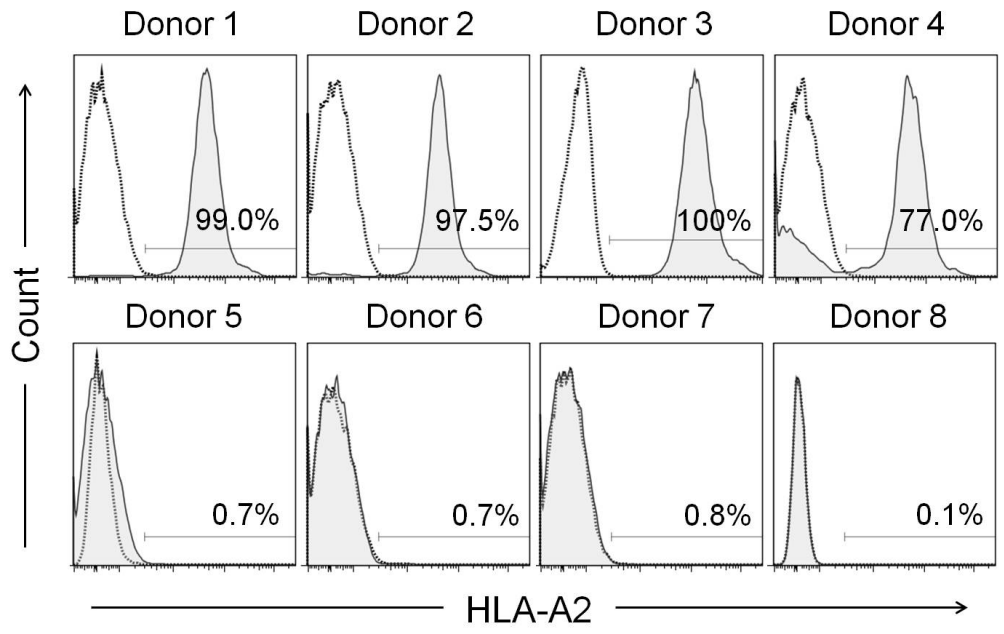


Figure 5. Haplotyping HLA-A2$^+$ donors. PBMCs isolated from whole blood of 8 individual healthy blood donors were immunostained for HLA-A2 using directly-conjugated anti-HLA-A2-PE antibody and analysed by flow cytometry. Unshaded and grey histograms represent unstained control and anti-HLA-A2 stained cells, respectively. Values in histograms represent percentages of positive cells.

3.2.2. Expression of surface CD137 corresponds to IFN-γ production by activated T cells

Activation of T cells is often noticeable by degranulation or by detection of intracellular cytokines such as IFN-γ. However, surface expression of CD137 has been well documented to be activation-dependent in both murine and human CD4$^+$ and CD8$^+$ T cells (Kwon and Weissman, 1989; Schwarz et al., 1993). A study by Wolfl et al. also showed that CD137 has favourable characteristics as a surrogate T-cell activation marker as it is expressed on nearly all responding cells regardless of their cytokine secretion profile (Wolfl et al., 2007). This finding was reaffirmed using PBMCs from a donor who is CMV$^+$ EBV$^-$. PBMCs were gated for CD3$^+$ cells and were analysed for the expression of intracellular IFN-γ or membrane-bound CD137 upon overnight

treatment with CMV-pp65 or EBV-LMP2A peptide-pools (figure 6). It is worthwhile to note that the peptides used in the experiment were commercial peptide-pools which consist mainly of 15-mer peptides with 11 amino acid overlaps, covering the complete sequence of the pp65 protein of human CMV or of the LPM2A protein of human EBV. Therefore, both antigen-specific $CD4^+$ and $CD8^+$ effector/memory T cells can be activated by the peptide-pool. T cells in unpulsed conditions or pulsed with LMP2A were in their resting state and therefore devoid of both IFN-γ and CD137 expression. The presence of positive-control CytoStim, an antibody-based reagent that acts similarly to a superantigen but independently of Vβ domains of the TCR and only in the presence of APCs, induced both IFN-γ and CD137 expression in both the $CD8^+$ and $CD8^-$ T-cell population. Similarly, in a pp65-pulsed condition, activation of antigen-specific T cells could be detected by using either IFN-γ or CD137 expression as a marker. More importantly, the degree of sensitivity was at least equal, if not higher when CD137 was used as an activation marker especially in the positive-control condition. Therefore, both IFN-γ and CD137 expression can be use as T-cell activation markers and will feature interchangeably throughout this section.

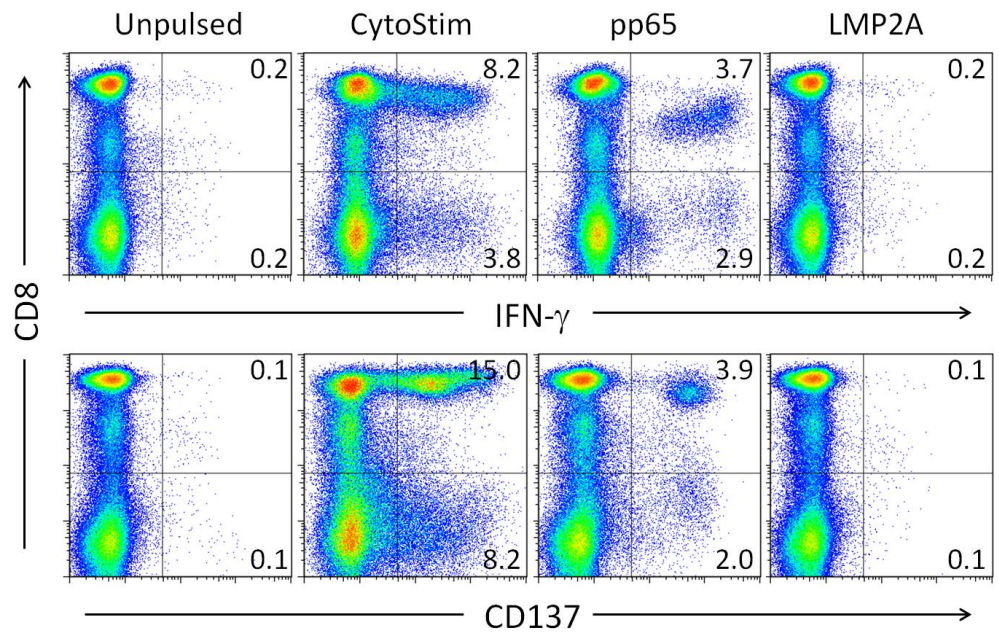


Figure 6. CD137 surface expression on T cells is an ideal surrogate marker of activation. PBMCs were isolated from a pp65^{+} LMP2A^{-} donor and were pulsed with either CMV pp65 or EBV LMP2A peptide pool. Unpulsed and CytoStim conditions acted as negative and positive controls, respectively. After 18 h of culture, cells were immunostained intracellularly for IFN-γ (top panel) or surface CD137 (bottom panel) and counterstained for CD3 and CD8. Depicted are flow cytometry histograms gated on the CD3^{+} population. Values in the histograms represent percentages of positive cells.

3.2.3. Antigen recall capacity in donor PBMCs

The ability of donors to respond to several different recall antigens was tested to determine the best candidates for subsequent studies involving CD137L-DCs. The virus-based CMV-pp65 and EBV-LMP2A peptide pools were utilised as antigens since latent infection is prevalent in 50-85 % and 80-90 %, respectively, in human adults thus significantly increasing the chance of acquiring a positive donor. Even though both viruses are not commonly considered as oncogenic viruses, barring the positive correlation of CMV with glioblastoma and EBV with nasopharyngeal carcinoma, utilising these viral-based antigens would simplify the antigen-specific experimental model. This will allow for the verification of our 'proof-of-concept' question in which we propose that CD137L-DCs are more potent APCs. Donor response towards the major melanoma-associated antigen MelanA/MART-1 peptide pool was also performed as high frequencies of MelanA/MART-1-specific naive T cells have been found in a large proportion of healthy individuals especially those who are HLA-$A2^+$ (Pittet et al., 1999).

PBMCs were pulsed with the various peptide pools and CD137 expression on T cells was analysed. Out of a total of 8 donors, 3 individuals (donors 1, 2 and 8) responded strongly to pp65 peptide pool restimulation while donor 4 was a weak responder (figure 7). Donor 2 was the only individual who responded strongly while donor 3 responded weakly to LMP2A antigen. None of the donors' PBMC tested were able to restimulate MelanA/MART-1 T cells as evidenced by the lack on $CD137^+$ T cells. As donors 1 and 2 were both HLA-$A2^+$ and could restimulate pp65 and/or LMP2A-specific T cells, they were recruited for further studies involving CD137L-DCs. Table 3 summarises the haplotype and responses of the 8 donors to the various peptide-antigens tested.

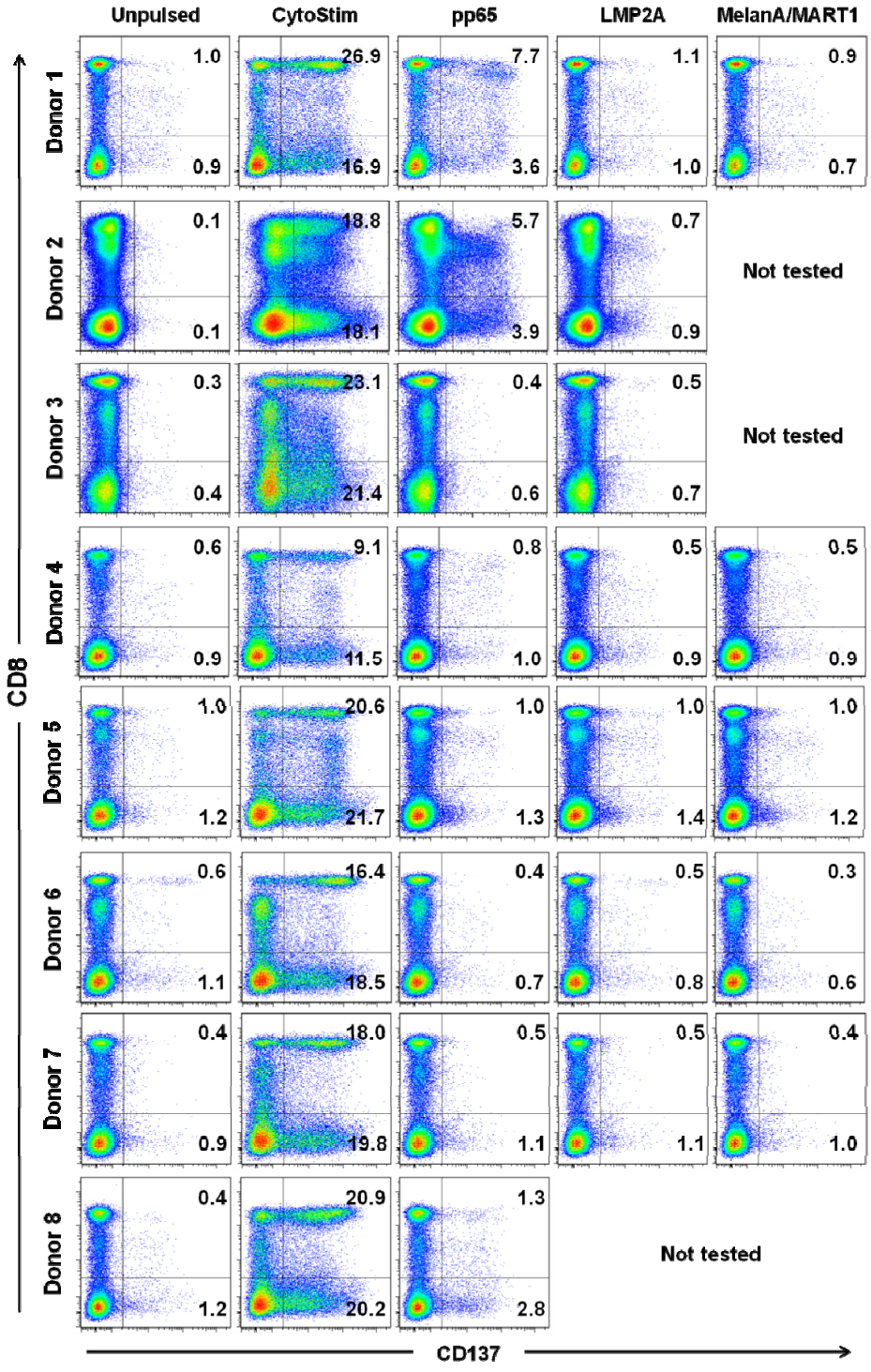


Figure 7. Antigen recall ability of donors. PBMCs isolated from whole blood of 8 individual healthy blood donors were pulsed with either CMV pp65, EBV LMP2A or melanoma MelanA/MART-1 peptide pool. Unpulsed and CytoStim conditions acted as negative and positive controls, respectively. After 18 h of culture, cells were immunostained for surface CD137 and counterstained for CD3 and CD8. Depicted are flow cytometry histograms gated on $CD3^+$ population. Donors 2, 3 and 8 were not tested for recall towards MelanA/MART-1 and donor 8 was not tested for recall towards LMP2A. Values in histograms represent percentages of positive cells.

Table 3. Donor haplotype and recall capacity to various peptide pool antigens.

Donor	Haplotype: HLA-A2	pp65	LMP2A	MelanA/ MART-1
1	✓	✓	×	×
2	✓	✓	✓	NT
3	✓	×	?	NT
4	✓	?	×	×
5	×	×	×	×
6	×	×	×	×
7	×	×	×	×
8	×	✓	NT	NT

✓ : positive, × : negative, ? : weak/uncertain, NT : not tested

3.2.4. Antigen recall assay using various DCs types (restimulation using CMV pp65 and EBV LMP2A peptide pools)

Having established the donor haplotype and responses towards various peptide antigens, the capacity of CD137L-DCs to restimulate antigen-specific T cells could be tested. In this instance, donors 1 and 2 were ideal candidates. Classical DCs and CD137L-DCs were generated over a period of 7 days and were pulsed with either the pp65 or the LMP2A peptide pool. Simultaneously, DCs were activated with either LPS or TNF overnight to induce further maturation followed by a co-culture with autologous pan T cells for 18 h. Fc-treated monocytes were not used as controls as the resultant cells are not able to survive well beyond 6 days and in fact, only less than 10 % are viable at day 10 (Langstein and Schwarz, 1999). Thus, it is impractical to use these Fc-treated monocytes in our experimental setting.

Despite restimulation by either classical DCs or CD137L-DCs, there were no significant differences in the percentages of activated pp65-specific T cells in

donor 1 (figure 8A). Additional maturation of DCs by either LPS or TNF was unable to heighten the activation of both $CD8^+$ and $CD8^-$ T cells. As expected, DCs pulsed with LMP2A were unable to activate any T cells as the donor was EBV^-. A different situation was observed in donor 2 where antigen-pulsed CD137L-DCs were slightly more potent than classical DCs (figure 8B). CD137L-DCs pulsed with pp65 were able to activate 2.7% of antigen-specific $CD8^+$ T cells while only 2.2% were activated when they were co-cultured with pp65-pulsed classical DCs. When comparing the population of $CD8^-$ T cells, CD137L-DCs and classical DCs activated 2.7% and 2.3% of antigen-specific T cells, respectively. Similarly, CD137L-DCs were capable of activating LMP2A-specific $CD8^+$ T cells (1.1%) and $CD8^-$ T cells (0.8%) whereas classical DCs could not activate them at all. Addition of LPS or TNF during the pulsing step failed to increase the capacity of CD137L-DCs to activate antigen-specific T cells.

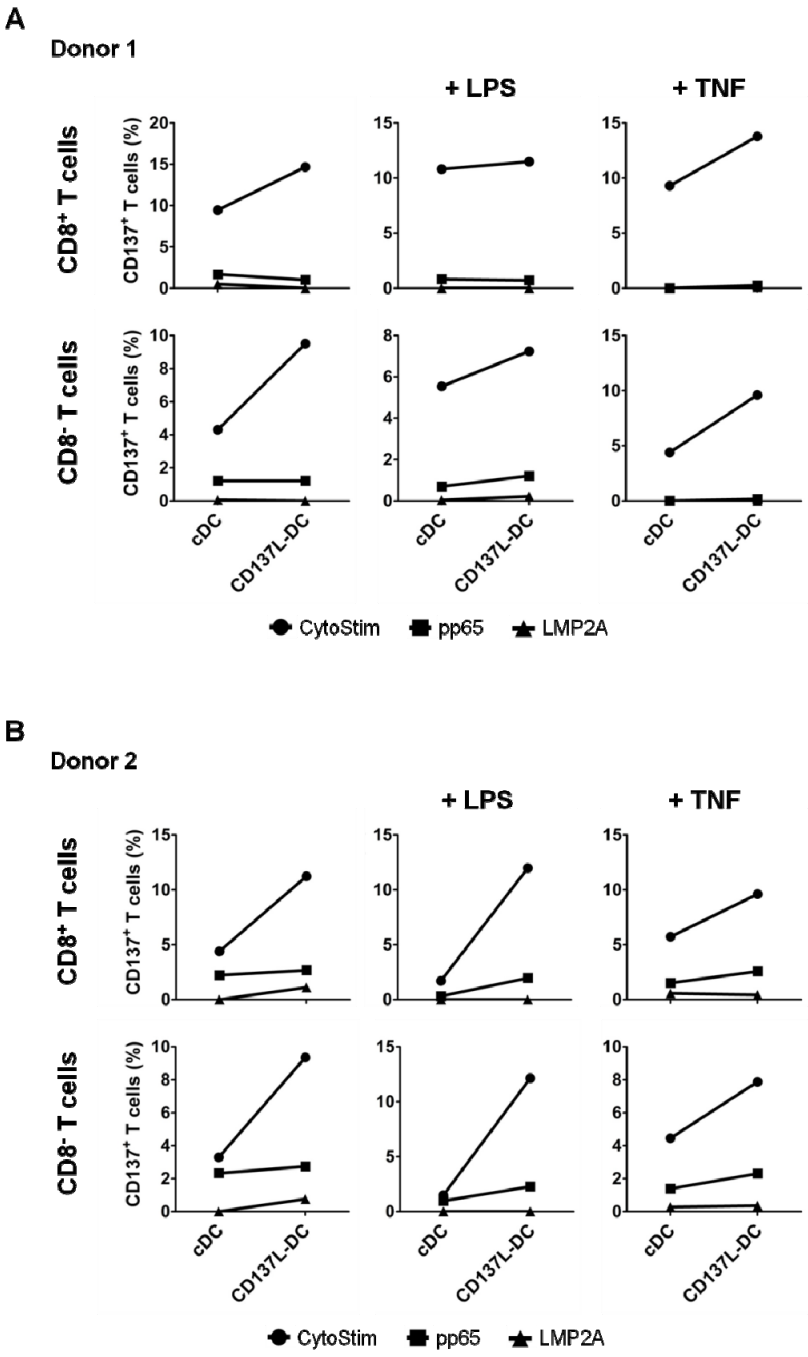


Figure 8. CD137L-DCs are able to activate antigen-specific T cells to a degree similar or slightly better than classical DCs. Classical DCs or CD137L-DCs generated from donors 1 (A) and 2 (B) were pulsed with either the CMV pp65 or the EBV LMP2A peptide pool and simultaneously co-cultured with autologous pan T cells at a ratio of 1:10. DCs pulsed with CytoStim condition acted as a positive control. In certain cases, DCs were treated with either LPS or TNF for 18 h prior to co-culture. After an 18 h co-culture, cells were immunostained for surface CD137 and counterstained for CD3 and CD8. Depicted are line graphs gated on $CD3^{+}CD8^{+}$ population (top panel) and $CD3^{+}CD8^{-}$ population (bottom panel).

Levels of IFN-γ secreted by activated T cells further support the notion of CD137L-DCs being potent APCs. Despite the absence of any differences in the expression levels of T-cell activation marker upon restimulation by classical DCs or CD137L-DCs in donor 1, there was a significant increase in IFN-γ production by T cells co-cultured with pp65 peptide pool-pulsed CD137L-DCs as compared to classical DCs. In this condition, there was a 2.5 times increase in IFN-γ levels (figure 9A). A similar trend was observed when DCs were pre-treated with either LPS or TNF. However, maturation of DCs with these factors decreased the overall activation of pp65-specific T cells as IFN-γ levels were lower than in T cells co-cultured with untreated DCs. Given that the donor was HLA-$A2^{+}$, the immunodominant NLVPMVATV (NLV) peptide derived from CMV pp65 protein was also utilised to evoke a response. Surprisingly, the absence of an increase in IFN-γ meant that the T cells were not activated by this particular peptide and perhaps the peptide-pool provided a variety of other peptides which were more dominant in evoking an immune response. As expected, there were no changes in IFN-γ levels in LMP2A-pulsed co-cultures as compared to unpulsed co-culture controls. A similar observation was made with donor 2 (figure 9B). The increased expression of membrane CD137 on T cells activated by CD137L-DCs corresponded to the higher IFN-γ secretion in their supernatant. These pp65 peptide pool-activated and LMP2A-activated T cells secreted 3.5 times and 1.5 times more IFN-γ, respectively, compared to their counterparts restimulated by classical DCs. As with donor 1, DCs matured with either LPS or TNF had an overall reduction in T-cell activation based on IFN-γ levels although CD137L-DCs were still more potent than their classical counterparts. Due to constrain in resources, NLV peptide was not used for donor 2.

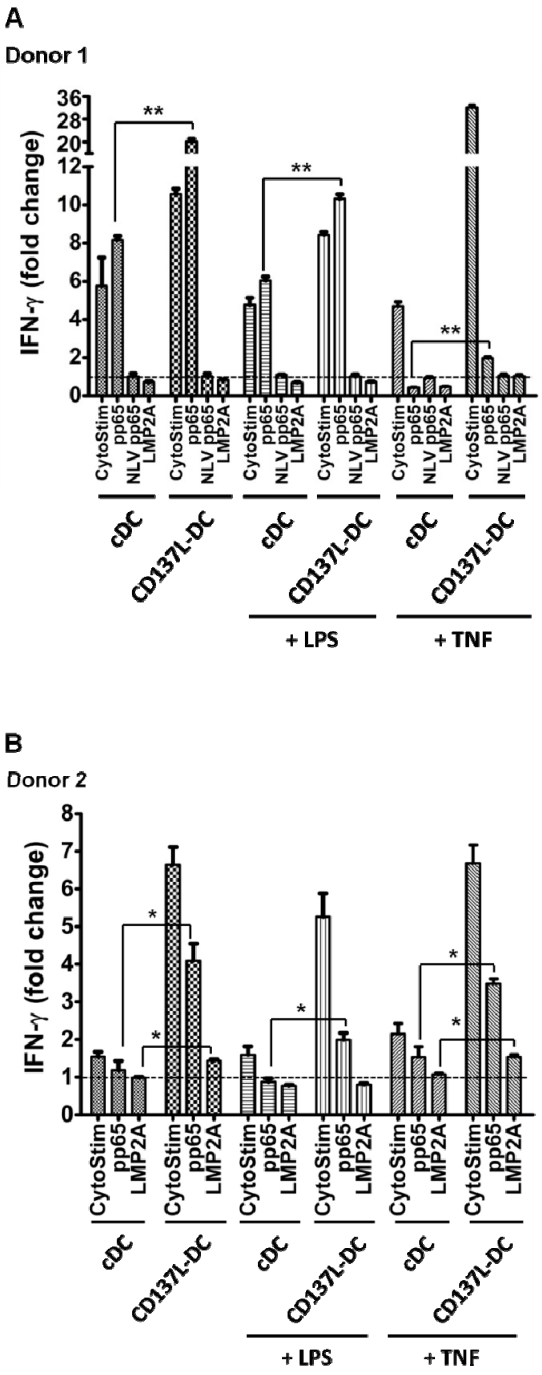


Figure 9. Antigen-specific T cells activated by CD137L-DCs secrete high amounts of IFN-γ. Classical DCs or CD137L-DCs generated from donors 1 (A) and 2 (B) were pulsed with either the CMV pp65 immunodominant peptide (NLV), CMV pp65 or the EBV LMP2A peptide pool, and simultaneously co-cultured with autologous T cells at a ratio of 1:10. DCs pulsed with CytoStim condition acted as a positive control. In certain conditions, DCs were activated with either LPS or TNF for 18 h prior to co-culture. Supernatants were harvested after 18 h of co-culture and concentrations of IFN-γ were determined by ELISA. Depicted are fold-changes of IFN-γ concentrations in relation to corresponding unpulsed conditions. Bar charts depict means ± standard deviations of triplicate measurements. *p<0.05; **p<0.01 using a two-tailed unpaired Student's t-test.

To further support the observation that CD137L-DCs are more potent APCs, T-cell proliferation was measured upon co-culture with antigen-pulsed DCs. Enhanced IFN-γ secretion by activated pp65 T cells was accompanied with enhanced cell proliferation (figure 10). Both, the pp65 peptide-pool and the NLV peptide were able to induce pp65 specific-T-cell proliferation although the former was clearly much more competent. Essentially, both types of DCs were able to efficiently induce strong pp65 T-cell proliferation (using pp65 peptide-pool) and there was no significant difference between them. The addition of LPS to further activate the DCs was not able to further enhance T-cell proliferation thus indicating that LPS was not a suitable maturation factor for either type of DCs. In fact, LPS treatment on classical DCs led to an increase in background T-cell proliferation even in the absence of peptide antigens. Due to constrain in resources, DCs from donor 2 were not tested for their ability to activate T cells.

Taken together, CD137L-DCs are able to present peptide antigens and activate autologous T cells in an antigen-specific manner. These T cells seemed to be more activated than those restimulated by classical DCs as evidenced by the slightly higher surface expression of the activation marker CD137 and profound elevation of IFN-γ secretion although there was no difference in proliferation rates.

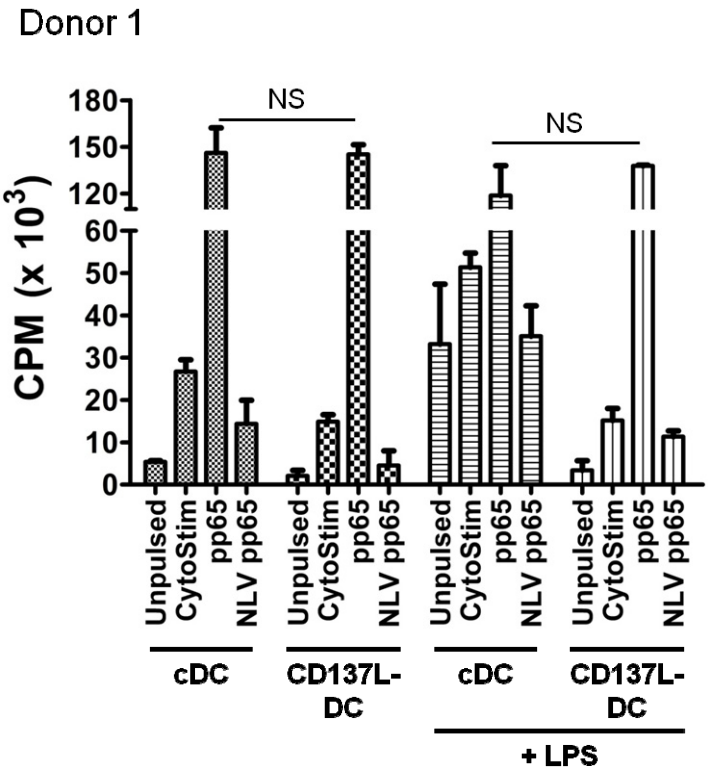


Figure 10. CD137L-DCs are not able to enhance the proliferative capacity of antigen-specific T cells. Classical DCs or CD137L-DCs generated from donor 1 were pulsed with either CMV pp65 immunodominant peptide (NLV) or the CMV pp65 peptide pool and simultaneously co-cultured with autologous T cells at a ratio of 1:10. DCs pulsed with CytoStim condition acted as a positive control. In indicated conditions, DCs were activated with either LPS for 18 h prior to co-culture. At day 5, proliferation was quantified by ^{3}H-thymidine incorporation. Proliferation was measured as counts per minute (CPM). NS = not significant.

3.2.5. Antigen recall assay using various DC types (restimulation using tetanus toxoid)

Besides using a peptide-based antigen, whole protein antigens from tumour lysates are also commonly used for DC-based immunotherapy (Cintolo et al., 2012). It is therefore appropriate that the ability of CD137L-DCs to process and present such antigens be determined. To investigate this, a recall assay using tetanus toxoid as a model antigen was performed on donors 1 and 3 who were recently immunized with tetanus vaccine (<3 years, personal communication with donors). As with the peptide-pool recall assay, the various DCs were pulsed with tetanus toxoid and simultaneously matured with LPS. After an overnight incubation, DCs were co-cultured with autologous pan T cells. The cytokine profile and proliferation rates were determined as a measure of tetanus toxoid-specific T-cell activation.

T cells from both donors produced high levels of cytokines when challenged with tetanus toxoid and this is consistent with them being recently vaccinated with tenatus toxoid (figure 11). Although both DC subsets were able to activate tetanus toxoid-specific T cells, classical DCs were more potent than CD137L-DCs as observed by the higher expression of cytokines by classical DC activated T cells. This is especially pronounced for IFN-γ whereby tetanus toxoid-specific T cells activated by classical DCs produced at least 3 times more cytokine than T cells activated by CD137L-DCs in both donors. Interestingly, maturation of classical DCs with LPS drastically reduced IFN-γ but increased IL-10 production which indicates a shift towards an anti-inflammatory profile. Such a response was not seen in LPS-treated CD137L-DCs suggesting that different TLR ligands may be required to further manipulate these DCs. Another cytokine that has been reported to be produced by tetanus toxoid-specific T cells is IL-17 (Brenchley et al., 2008;

Lenarczyk et al., 2000). Indeed, there was a dose-dependent increase of IL-17 by T cells from donor 1 but not from donor 3 and this could be contributed to donor variation. Nonetheless, CD137L-DCs were unable to cultivate a stronger antigen-specific response which is in contrast to the previous finding whereby these DCs were able to heighten IL-17 expression more potently than their classical counterparts (Kwajah and Schwarz, 2010).

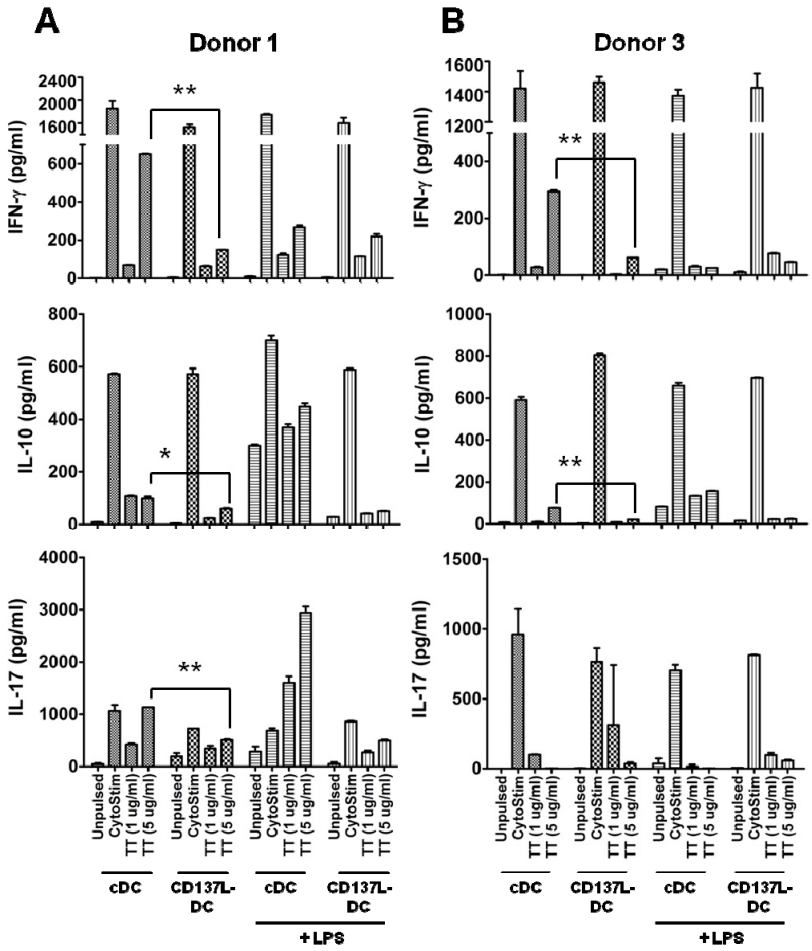


Figure 11. T cells restimulated by classical DCs secrete more cytokines in response to whole protein antigens. Classical DCs or CD137L-DCs generated from donors 1 (A) and 3 (B) were pulsed with increasing concentrations of tetanus toxoid and were co-cultured with autologous T cells at a ratio of 1:10. DCs pulsed with CytoStim condition acted as a positive control. In certain conditions, DCs were activated with LPS for 18 h prior to co-culture. Supernatants were harvested after 6 days co-culture and concentrations of IFN-γ, IL-10 and IL-17 were determined by ELISA. Bar charts are illustrated as means ± standard deviations of triplicate measurements. *p<0.05; **p<0.01 using a two-tailed unpaired Student's t-test.

The proliferative capacity of tetanus toxoid-specific T cells ran in parallel with their IFN-γ production whereby it followed a dose-dependent trend with respect to the amount to pulsed tetanus toxoid (figure 12). When compared between the different types of DCs pulsed with 1 μg/ml tetanus toxoid, classical DCs were more potent activators of antigen-specific T cells in both donors. However, with a higher concentration of pulsed antigen, both classical DCs and CD137L-DCs were able to induce similar rates of T-cell proliferation. LPS-treated DCs failed to further potentiate tetanus-toxoid T-cell proliferative capacity and this mirrors the previous finding when peptide antigens were utilised (section 3.2.4). Instead, these DCs and in particular classical DCs, were able to highly activate large numbers of non-specific T cells as evinced by the high T-cell proliferation in the unpulsed condition.

Collectively, although the data demonstrates that CD137L-DCs are able to stimulate and activate autologous T cells in an antigen-specific manner, to claim that CD137L-DCs are more potent APCs than classical DCs may be premature due to a minimal increase in the T-cell activation marker and a similar potency to classical DCs in inducing T-cell proliferation in a peptide recall assay. Furthermore, classical DCs seemed to be more efficient APCs in the presence of whole protein antigens. Disappointingly, the addition of either LPS or TNF to mature CD137L-DCs was unable to further enhance T-cell activation, and this is in line with previous data from our laboratory (Kwajah and Schwarz, 2010). Thus, before embarking on further antigen-specific experiments, improvements in terms of maturing CD137L-DCs has to be carried out to further increase the potency of CD137L-DCs to initiate optimal T-cell activation.

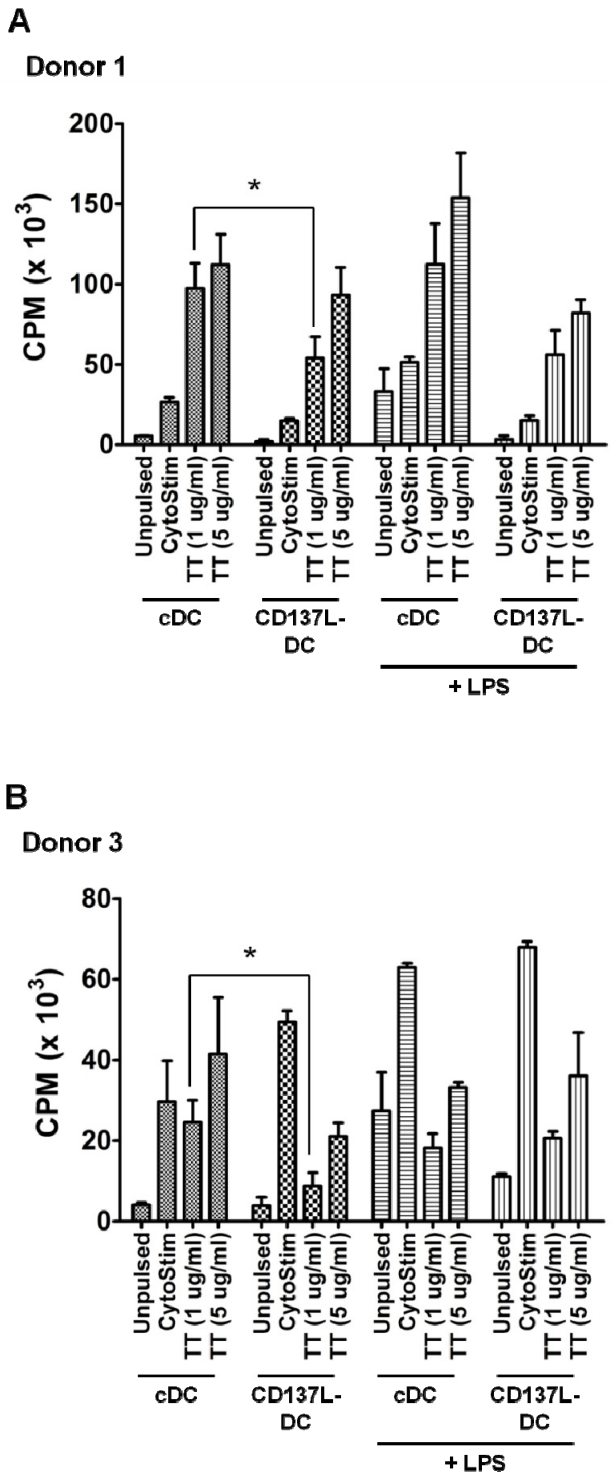


Figure 12. T cells restimulated by classical DCs have a higher proliferative capacity in response to whole protein antigens. Classical DCs or CD137L-DCs generated from donors 1 (A) and 3 (B) were pulsed with tetanus toxoid at increasing concentrations and were co-cultured with autologous T cells at a ratio of 1:10. DCs pulsed with CytoStim condition acted as a positive control. In indicated conditions, DCs were activated with either LPS for 18 h prior to co-culture. At day 5, proliferation was quantified by ^{3}H-thymidine incorporation. *$p<0.05$ using a two-tailed unpaired Student's *t*-test. CPM = counts per minute.

3.3. Maturation of CD137L-DCs

The recall assay experiments presented in the previous section showed that CD137L-DCs are indeed able to function as an APC and activate T cells in an antigen-specific manner. Nevertheless, the potential to further mature these DCs exist as it was previously shown that the expression of DC maturation markers such as CD83, CD86 and HLA-DR can be enhanced by LPS and IFN-γ although the capacity to elicit a T-cell response was not affected (Kwajah and Schwarz, 2010). Likewise, maturation with either LPS or TNF alone as discussed in the previous section, proved to be redundant or insufficient. Hence, in this section we discuss methods to mature CD137L-DCs with the eventual aim of eliciting an even more potent activated T-cell status.

In the previous study by Kwajah and Schwarz, they termed CD137L-DCs as already having a mature phenotype based on the presence of CD83 and their ability to activate allogeneic naive and memory T cells to levels superior than mature classical DCs (Kwajah and Schwarz, 2010). Due to this reason, we refrain from calling CD137L-DCs that are not treated with any exogenous maturation factors as 'immature CD137L-DCs' but rather as 'untreated CD137L-DCs'.

Several well-established maturation factor cocktails were tested for their ability to mature CD137L-DCs (table 4). These include the standard LPS plus IFN-γ and the gold standard Jonuleit cocktail for immunotherapy, comprising of TNF, IL-1β, IL-6 and PGE2. CD137L-DCs were generated for 7 days, followed by maturation with the various cocktails during the final 18 h of culture.

Table 4: Cytokine combinations employed for CD137L-DC maturation

Cocktail	Components (concentration)		Remarks
C0	-	-	Untreated CD137L-DCs
C1	LPS IFN-γ	1 μg/ml 50 ng/ml	IL-23 control for CD137L-DCs (Kwajah and Schwarz, 2010)
C2	TNF IL-1β IL-6 PGE2	10 ng/ml 10 ng/ml 10 ng/ml 1 μg/ml	Jonuleit cocktail (Jonuleit et al., 1997)
C3	TNF IL-1β PGE2 IFN-γ R848	10 ng/ml 10 ng/ml 250 ng/ml 250 ng/ml 1 μg/ml	Zobywalski cocktail 1 (Zobywalski et al., 2007)
C4	TNF IL-1β PGE2 IFN-γ R848 Poly (I:C)	10 ng/ml 10 ng/ml 250 ng/ml 250 ng/ml 1 μg/ml 100 ng/ml	Zobywalski cocktail 2 (Zobywalski et al., 2007)
C5	R848 Poly (I:C)	3 μg/ml 100 ng/ml	Boullart cocktail 1 (Boullart et al., 2008)
C6	Flagellin	25 ng/ml	TLR5 agonist (low concentration)
C7	Flagellin	50 ng/ml	TLR5 agonist (high concentration)

3.3.1. Effects of maturation cocktails on CD137L-DC phenotype

Morphological differences were observed upon the treatment of CD137L-DCs with the various maturation cocktails (figure 13). C0 (untreated) CD137L-DCs is a mixed population of cells with fiber-like extensions and spindle-shaped cells which are strongly adhered to the culture plate, and floating cells with rounded morphologies. Upon 18 h treatment with either C3 or C4, most of the floating cells became adherent while those already attached became flatter and apparently even more strongly adherent. These cells also had more pronounced extension and spindles. This increase in adherence was made obvious when the cells were harvested. In the presence of trypsin-EDTA, twice the time was required to completely detach the C3 or C4-treated cells

from the culture plate as compared to the untreated CD137L-DCs (figure 14). Despite any clear morphological distinction between untreated CD137L-DCs and those treated with C1, C2, and C5, the adherent properties differed amongst these differentially treated DCs. C1 and C5-treated CD137L-DCs were more adherent than untreated CD137L-DCs while the opposite is true for C2-treated DCs. This reduced adherence is likely due to the presence of PGE2 in the cocktail which has been reported to reduce attachment properties and therefore favour migration (Boullart et al., 2008).

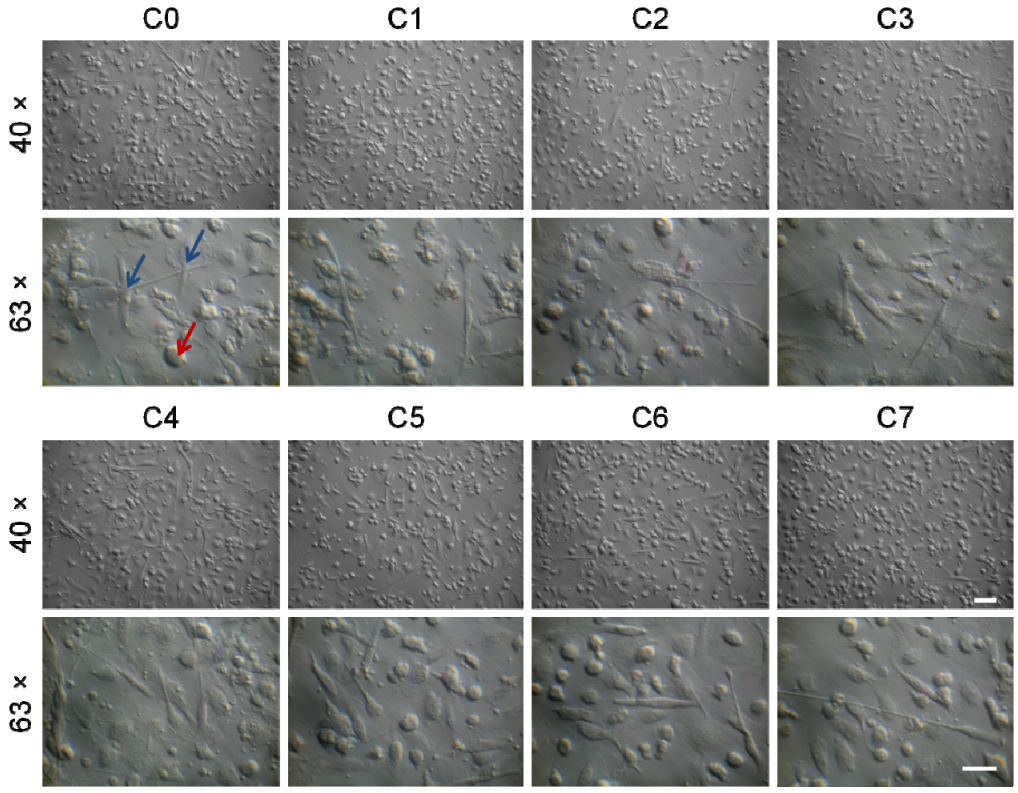


Figure 13. Maturation cocktails C3 and C4 induced morphological changes in CD137L-DCs. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Photographs were taken on day 7 at magnification of 40 and 63 ×. Blue arrows refer to attached cells while red arrows refer to floating cells. Scale bar: 20 μm

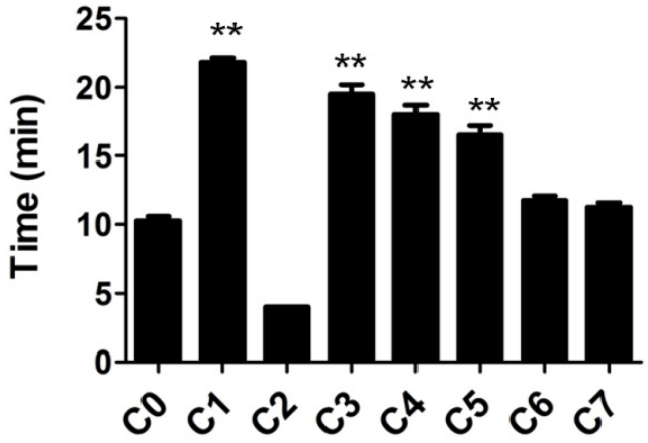


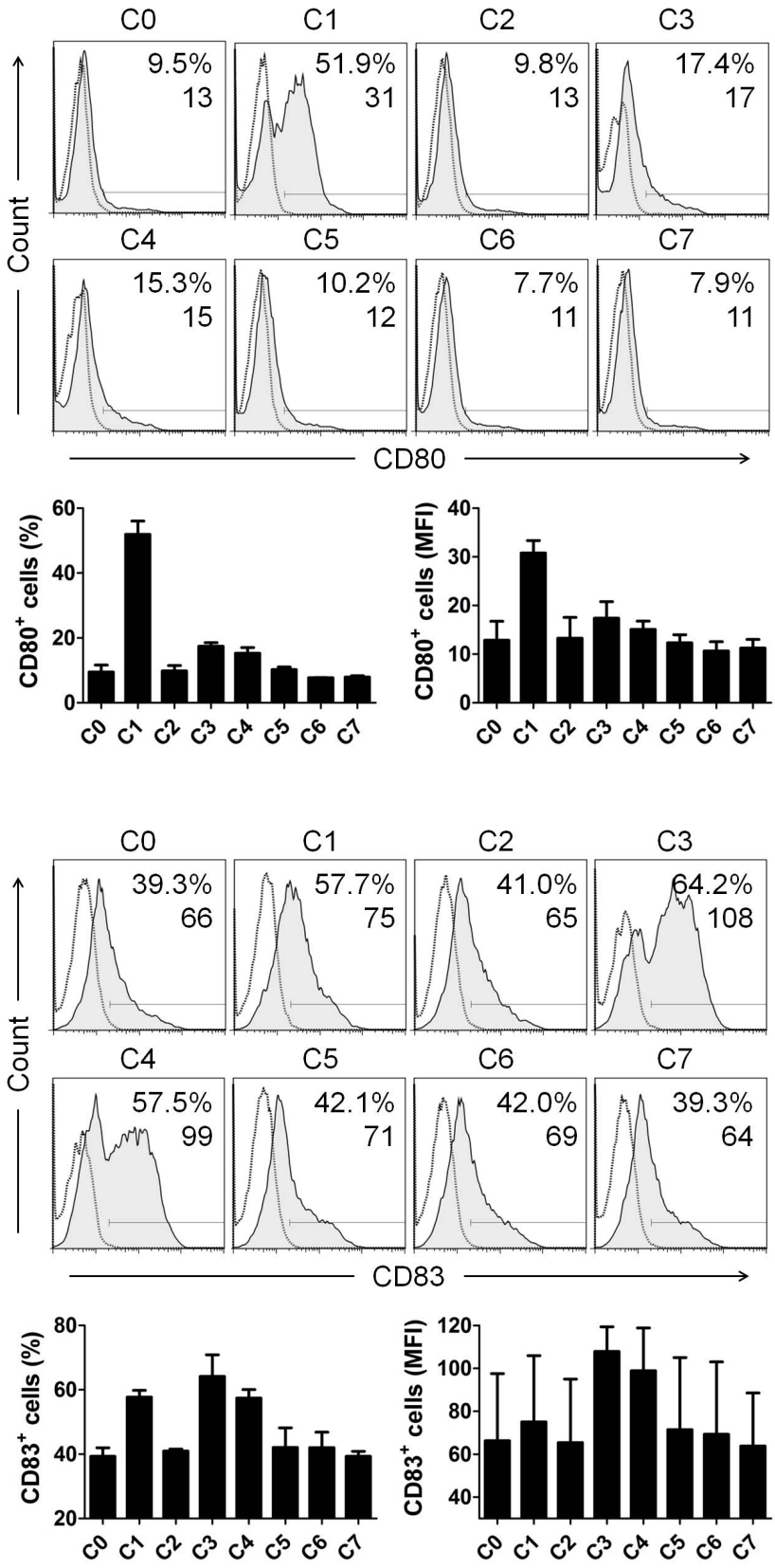
Figure 14. Maturation cocktails C1, C3, C4 and C5 are able to increase adhesion properties of CD137L-DCs. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Cells were washed and treated with trypsin-EDTA with constant agitation to allow cells to detach. Depicted are times taken for cells to completely detach from wells as observed by microscopy. Bar charts depict means ± standard deviations of triplicate measurements. $^{**}p<0.01$ using a two-tailed unpaired Student's *t*-test. This experiment has been performed twice with comparable results.

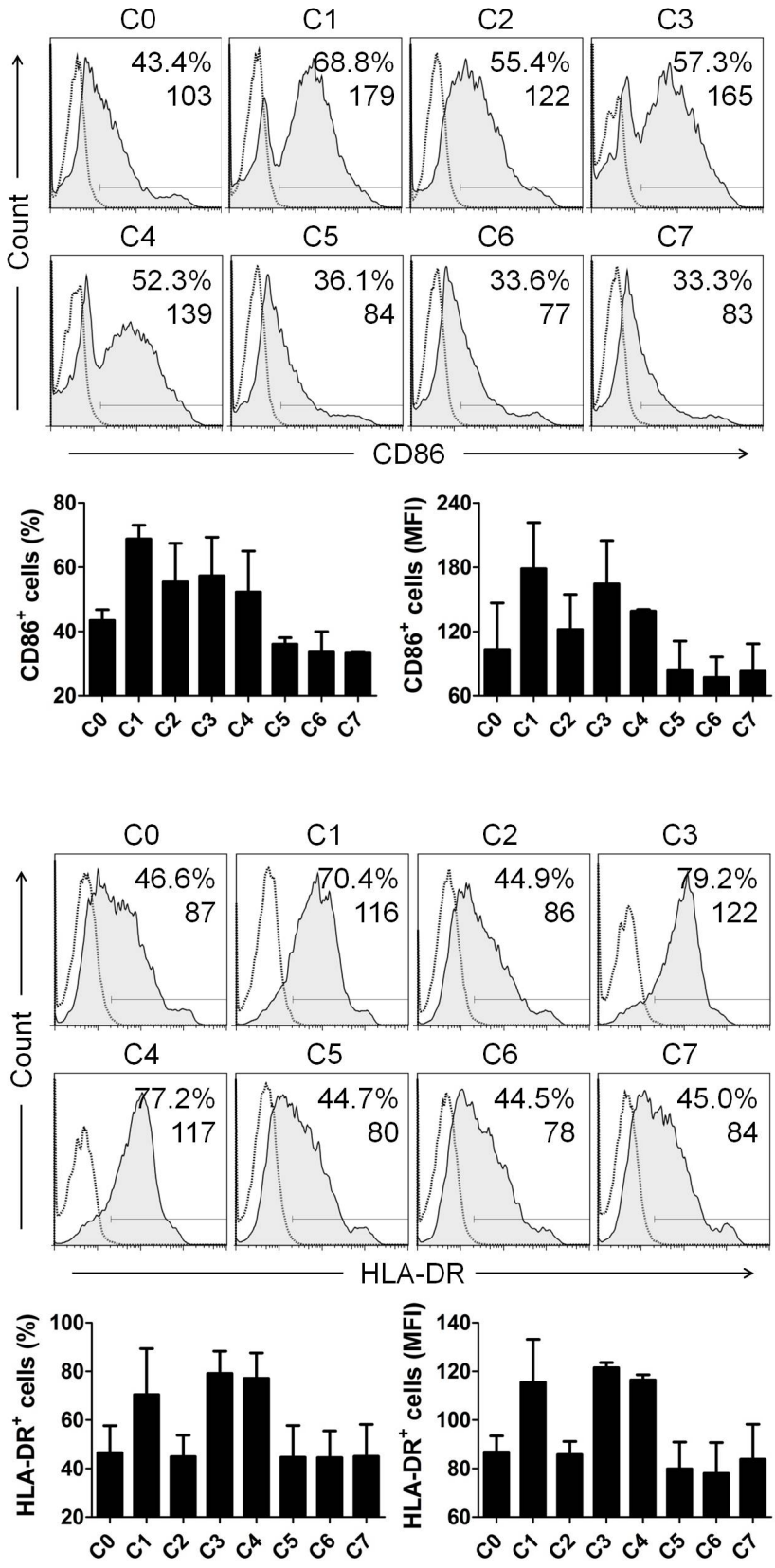
Further, the expression profile of DC surface protein markers was determined to gauge the level of DC maturation (figure 15). Basal levels of the DC maturation marker CD83 were expressed by 39 % of untreated CD137L-DCs, with a MFI of 66. This was significantly enhanced by C3 (64 %, MFI = 108) and C4 (58 %, MFI = 99) while C1 increased expression only modestly (58 %, MFI = 75). Treatment by cocktails C2, C5, C6 and C7 had no effect on CD83 expression. A similar pattern was observed for the co-stimulatory molecule CD86 (B7-2). 43 % of untreated CD137L-DCs expressed CD86, with an MFI of 103. Treatment of CD137L-DCs with C1, C3 and C4 led to a considerable upregulation of CD86 (69 %, MFI = 179; 57 %, MFI = 165; 52 %, MFI = 139, respectively) while only a slight increase was observed with C2 (55 %, MFI = 122). The expression of the other major member of the B7 family, CD80, was

also enhanced by cocktails C1, C3 and C4 albeit at much lower levels by cocktails C3 and C4 (figure 15).

MHC class II is required for the presentation of antigen epitopes and the subsequent activation of antigen-specific autologous $CD4^+$ T cells. It was therefore surprising that CD137L-DCs, despite being potent activators of $CD4^+$ T cells, expressed low levels of MHC class II (Kwajah and Schwarz, 2010). Thus, we hypothesised that an increase in surface MHC class II expression on CD137L-DCs would be one of the most likely events that can enhance the activation of $CD4^+$ T cells by CD137L-DCs. Treatment with C1, C3 or C4 significantly enhanced levels of HLA-DR (70 %, MFI = 116; 79 %, MFI = 122; 77 %, MFI = 117, respectively) as compared to untreated CD137L-DCs (46%, MFI = 87), (figure 15).

Maturation is associated with the increased expression of co-stimulatory molecules, surface MHC class II as well as the acquisition of chemokine receptors such as CCR7 which allows the migration of DCs into the draining lymph node where they interact with T cells (Trombetta and Mellman, 2005). Indeed, treatment of CD137L-DCs with C1, C3, C4 and C6 increased CCR7 expression. However, treatment by C3 and C4 marginally reduced the expression of another chemokine receptor, CXCR4 (figure 15).


C0
C1
C2
C3
9.5%
13
51.9%
31
9.8%
13
17.4%
17
C4
C5
C6
C7
15.3%
15
10.2%
12
7.7%
11
7.9%
11
Count
CD80
CD80+ cells (%)
CD80+ cells (MFI)
39.3%
66
57.7%
75
41.0%
65
64.2%
108
57.5%
99
42.1%
71
42.0%
69
39.3%
64
CD83
CD83+ cells (%)
CD83+ cells (MFI)


C0
C1
C2
C3
43.4%
103
68.8%
179
55.4%
122
57.3%
165
C4
C5
C6
C7
52.3%
139
36.1%
84
33.6%
77
33.3%
83
Count
CD86
CD86+ cells (%)
CD86+ cells (MFI)
C0
C1
C2
C3
46.6%
87
70.4%
116
44.9%
86
79.2%
122
C4
C5
C6
C7
77.2%
117
44.7%
80
44.5%
78
45.0%
84
Count
HLA-DR
HLA-DR+ cells (%)
HLA-DR+ cells (MFI)

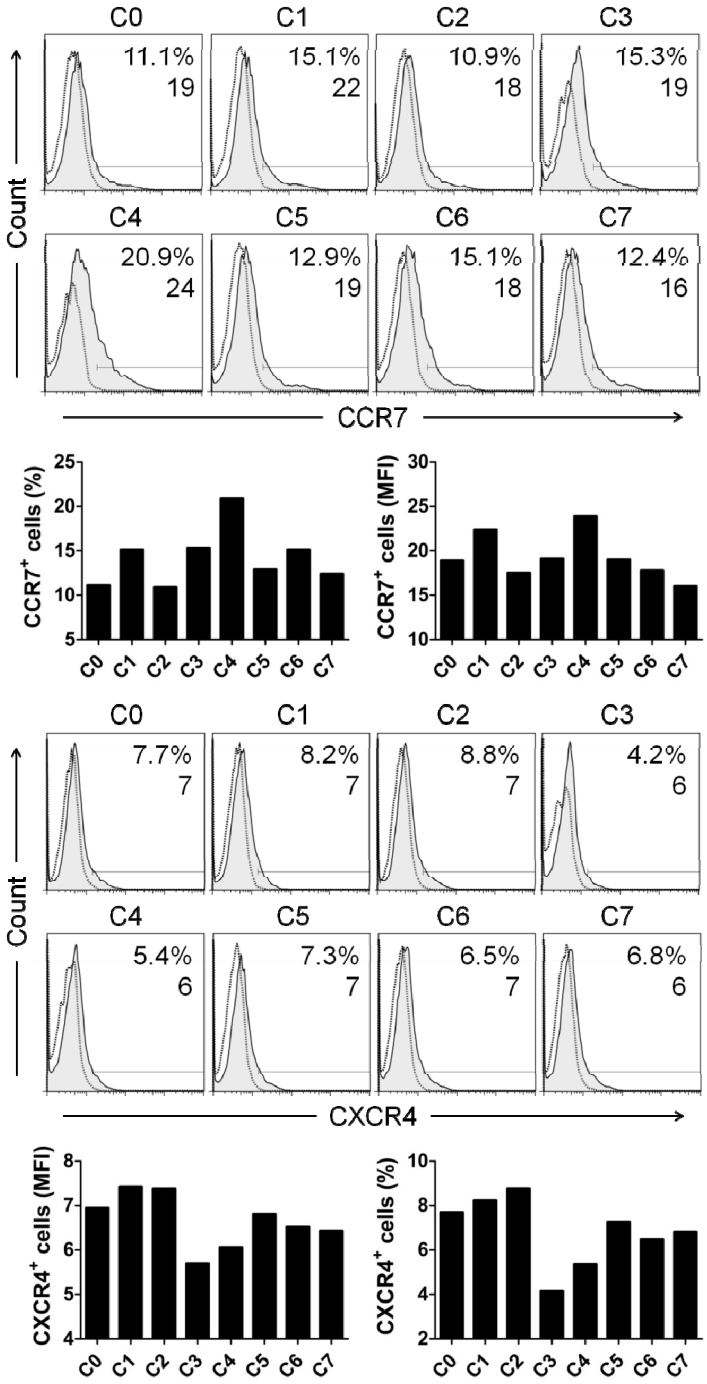


Figure 15. Maturation cocktails C1, C3 and C4 upregulates maturation markers on CD137L-DCs. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Cells were immunostained for CD80, CD83, CD86, HLA-DR, CCR7 and CXCR4 and analysed by flow cytometry. Unshaded and grey histograms represent unstained control and indicated surface markers, respectively. Values in histograms are percentages of positive cells and mean flourescence intensities (MFI), respectively. Bar charts on the bottom depict mean percentages and the mean MFI ± standard deviations of data from two independent donors, except for CCR7 and CXCR4, which are from a single donor.

DCs are the mediators between innate and adaptive immunity and often ensure the development of appropriate immune responses. The ability of DCs to polarise specific T-cell response is highly dependent on the cytokine profile of the DCs. Often critical for DC immunotherapy is the generation of Th1-polarising DCs in which IL-12 is a key cytokine that defines this DC phenotype. C1-treated CD137L-DCs produced more IL-12p40 than its untreated counterpart whereas treatment with C2 cocktail caused a slight reduction in cytokine expression (figure 16). The rest of the maturation cocktails had no effects on IL-12p40 production. Disappointingly, expression of functional IL-12p70 was absent in all conditions. Despite the lack of Th1-driving IL-12p70 cytokine, IL-23, which is a pro-inflammatory cytokine from the IL-12 family, was detected in supernatants from C1, C3, C4 and C5-treated CD137L-DCs.

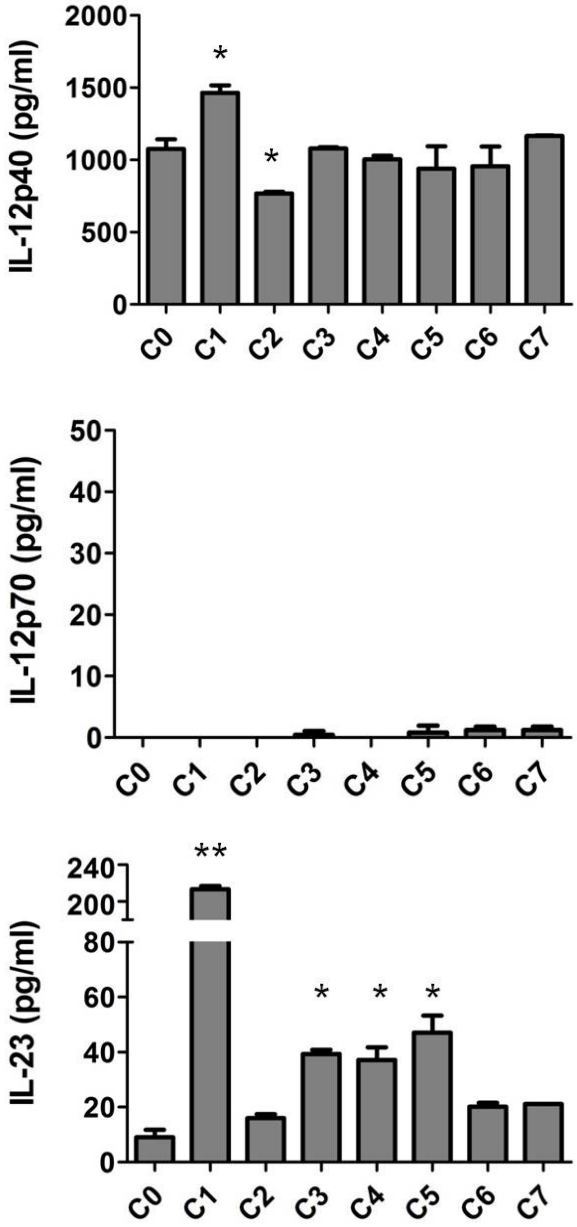


Figure 16. Maturation cocktails do not induce IL-12p70 while C1, C3, C4, and C5 promote IL-23 expression. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Supernatants were then harvested and concentrations of IL-12p40, IL-12p70 and IL-23 were determined by ELISA. Bar charts depict means ± standard deviations of triplicate measurements. *p<0.05; **p<0.01 using a two-tailed unpaired Student's t-test.

3.3.2. Effects of maturation cocktail treated CD137L-DCs on T-cell activities

Morphological, surface marker and cytokine profiles point towards a positive influence of cocktails C1, C3 and C4 to mature CD137L-DCs. However, to ascertain whether these maturation cocktails not only enhance expression of T-cell activating molecules but also enhance their overall T-cell activating potency, the ability of cocktail-treated CD137L-DCs to induce T-cell proliferation in an allogeneic MLR was assessed.

CFSE-stained allogeneic T cells were co-cultured with C1, C3 or C4-treated CD137L-DCs for 5 days and T-cell proliferation was determined by CFSE dilution. In the absence of CD137L-DCs, T cells remained resting as there was barely any proliferation (2 %, MFI = 1877), (figure 17A). Untreated CD137L-DCs were able to induce proliferation in 64 % of the T cells (MFI = 733), while C1-treated CD137L-DCs, despite having enhanced CD83, CD86 and HLA-DR expression, were not able to increase T-cell proliferation any further (68 % proliferation, MFI = 665). This confirmed the previous observation whereby the treatment of CD137L-DCs with LPS plus IFN-γ has no substantial effect on their ability to induce allogeneic T-cell responses (Kwajah and Schwarz, 2010). Nonetheless, CD137L-DCs treated with either C3 or C4 were more potent T-cell activators as evidenced by the enhanced T-cell proliferation. 81 % (MFI = 444) and 80 % (MFI = 463) of the T cells had undergone proliferation when co-cultured with C3 and C4-treated CD137L-DCs, respectively.

As an additional measure of CD137L-DC potency and as an initial indication of the ability to induce Th1 response, levels of IFN-γ in day 5 co-culture supernatants were determined. IFN-γ secreted by T cells co-cultured with C3-

treated CD137L-DCs were five times higher (1265 pg/ml ± 243 pg/ml) than those from control CD137L-DCs (241 pg/ml ± 74 pg/ml) while levels were three times higher (720 pg/ml ± 241 pg/ml) in supernatants of T cells co-cultured with C4-treated CD137L-DCs (figure 17B). This observation suggests a correlation between IFN-γ secretion with enhanced proliferation rate of T cells. While maturation with cocktails C1, C3 and C4 augmented DC activation markers expression, only C3 and C4 were able to boost the ability of CD137L-DCs to enhance T-cell proliferation and IFN-γ secretion.

Maturation cocktails C3 and C4 differ by the presence of poly (I:C) in C4 (table 4). Poly (I:C) is a TLR3 agonist with the ability to induce production of IL-12p70 in classical DCs (Boullart et al., 2008). However, CD137L-DCs treated with C4 containing poly (I:C) were not able to produce detectable IL-12p70 in the supernatants, nor were any of other maturation cocktails (figure 16). Since the presence of poly (I:C) did not contribute to CD137L-DC maturation, the other components, i.e. cocktail C3, mediated the functional maturation of CD137L-DCs.

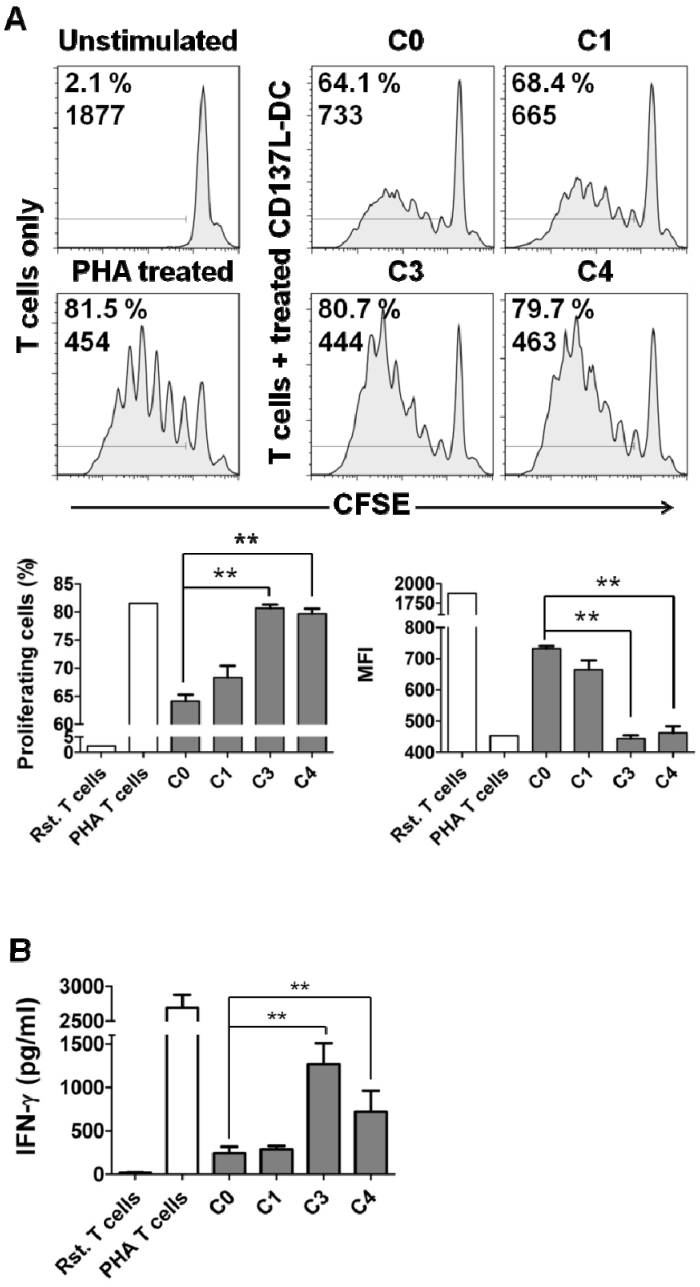


Figure 17. CD137L-DCs treated with either C3 or C4 are more potent T-cell activators. Monocytes were treated with immobilised CD137-Fc protein for 6 days and were further treated with the indicated maturation cocktails for 18 h. CD137L-DCs were then further co-cultured for 5 days with CFSE-stained allogeneic T cells at a ratio of 1:10. (A) Levels of T-cell proliferation were quantified by flow cytometry based on CFSE dilution. Values in the histograms and bar charts represent mean percentages of T cells undergoing CFSE-dilution and corresponding MFI of duplicate measurements. (B) Supernatants of day 5 co-cultures of maturation cocktail-treated CD137L-DCs with allogeneic T cells were harvested and levels of IFN-γ were determined by ELISA. Depicted are means ± standard deviations of triplicate measurements. ***p*<0.01 using a two-tailed unpaired Student's *t*-test. This experiment has been performed twice with comparable results. Rst. = resting; PHA = phytohaemagglutinin

3.3.3. R848 and IFN-γ are essential and sufficient to functionally mature CD137L-DCs

Having identified C3 as the most potent and suitable cocktail for maturing CD137L-DCs, the next aim was to deduce the essential components of this cocktail. C3 consist of five factors; IL-1β, TNF, IFN-γ, PGE2 and R848. Two of these inflammatory cytokines, IL-1β and TNF, were previously shown to be produced upon reverse signalling through CD137L in monocytes (figure 4). It is therefore reasoned that these components can be omitted from maturation cocktail C3. Additionally, we aim to systematically eliminate individual components of C3 in order to define a maturation cocktail that combines highest potency with the least components as it would aid future clinical use of CD137L-DCs (table 5).

Table 5: Definition of essential factors for CD137L-DC maturation.

Cocktail	Components (concentration)		Remarks
C0	-	-	Untreated CD137L-DCs
C3	TNF IL-1β PGE2 IFN-γ R848	10 ng/ml 10 ng/ml 250 ng/ml 250 ng/ml 1 μg/ml	Complete C3 cocktail.
C3A	PGE2 IFN-γ R848	250 ng/ml 250 ng/ml 1 μg/ml	Without TNF and IL-1β which is endogenously produced by CD137L-DCs (figure 4)
C3B	IFN-γ R848	250 ng/ml 1 μg/ml	-
C3C	IFN-γ	250 ng/ml	-
C3D	R848	1 μg/ml	-

As previously described, C3 induced a change in cellular morphology whereby CD137L-DCs became more attached and had more pronounced extension and spindles. In all but one derivatives of C3, such changes were similarly observed. The C3D cocktail, which only contained the TLR7/8 ligand R848, was unable to bring about a morphological change to CD137L-DCs, however, IFN-γ alone (C3C) was sufficient (figure 18).

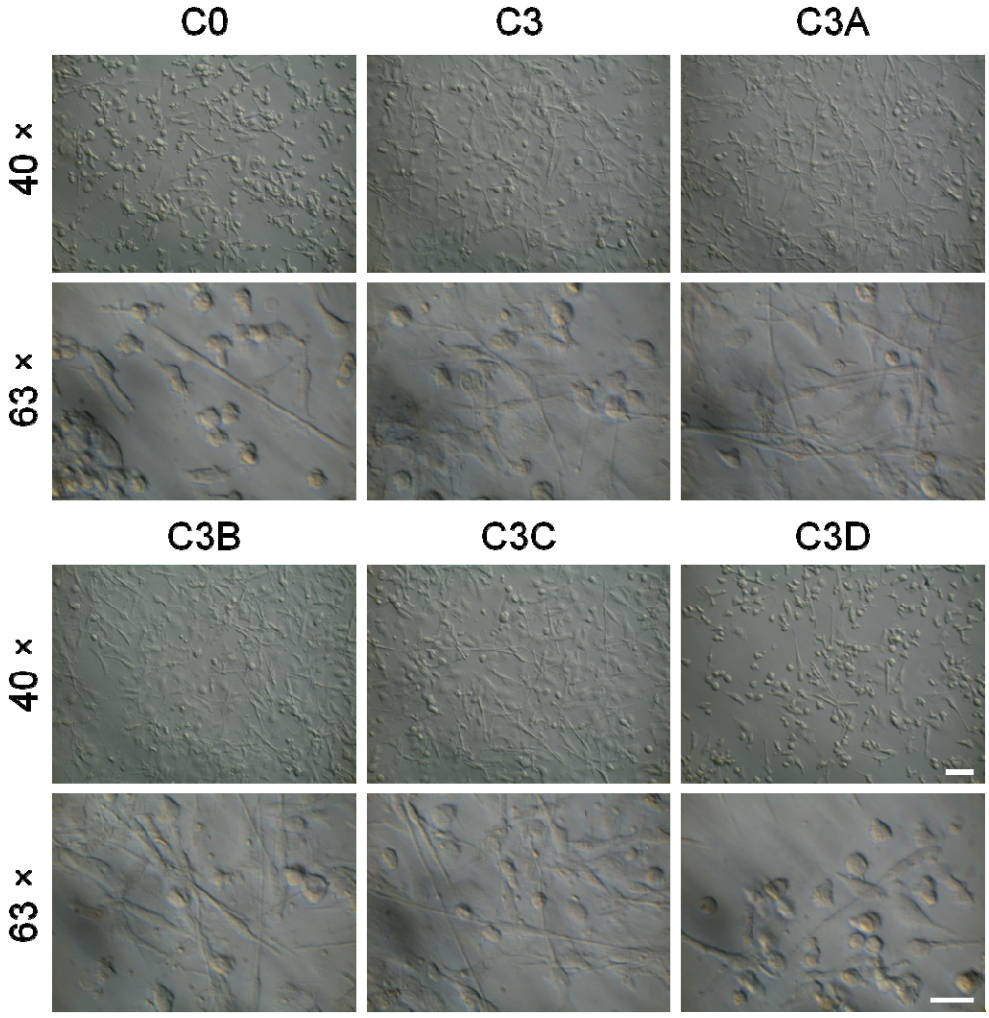
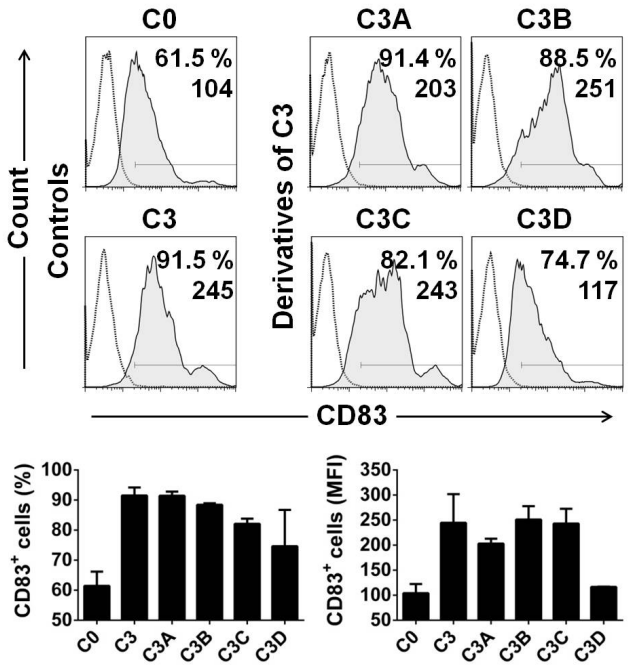


Figure 18. All derivatives of cocktail C3 induced morphological changes in CD137L-DCs except for C3D. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Photographs were taken on day 7 at magnification of 40 and 63 ×. Scale bar: 20 μm

As cocktail C3 was able to significantly upregulate the expression of CD83, CD86 and HLA-DR (figure 15), these DC maturation markers on CD137L-DC treated with C3-derived cocktails were also tested. The absence of IL-1β and TNF in cocktail C3A made no difference to the induction of CD83, CD86 and HLA-DR when compared to the parental C3 (figure 19). Similarly, production of IL-23 was equally upregulated with either C3 or C3A (figure 20). Besides, when T cells were co-cultured with C3 or C3A-treated CD137L-DCs, there were no differences in their proliferation rates and secretion of IFN-γ suggesting that the induction of endogeneous IL-1β and TNF by CD137L reverse signalling in monocytes and developing CD137L-DCs is sufficient to promote their maturation (figure 21).

On top of promoting a morphological change in CD137L-DCs, IFN-γ alone (C3C) was sufficiently able to increase the expression of CD83, CD86 and HLA-DR, and it also enhanced T-cell proliferation to similar levels as C3 (figures 19 and 21A). However, C3C-treated CD137L-DCs were unable to significantly raise IL-23 production and furthermore, it was inadequate to bring about a further increase in IFN-γ production by T cells in a co-culture condition (figures 20 and 21B). The TLR7/8 ligand R848 alone (C3D) was not able to increase expression of the co-stimulatory molecules and IL-23 but it was mildly sufficient to activate CD137L-DCs to a level where they could enhance T-cell proliferation and IFN-γ production (figures 19-21). Yet, the combination of R848 and IFN-γ (C3B) was as potent as the parental C3 cocktail in maturing CD137L-DCs as evidenced by the comparable phenotypic and functional competence of the DCs upon treatment by either maturation cocktails (figure 19-21). This demonstrates that R848 plus IFN-γ (C3B) is essential and sufficient for the full maturation of CD137L-DCs.


C0
C3A
C3B
61.5 %
104
91.4 %
203
88.5 %
251
Count
Controls
Derivatives of C3
C3
C3C
C3D
91.5 %
245
82.1 %
243
74.7 %
117
CD83
CD83+ cells (%)
CD83+ cells (MFI)
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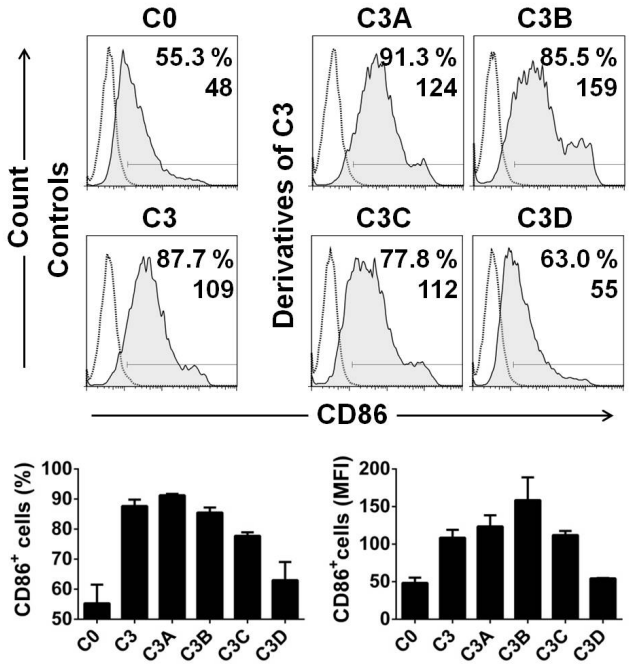
C3A
C3B
C3C
C3D


C0
C3A
C3B
55.3 %
48
91.3 %
124
85.5 %
159
Count
Controls
Derivatives of C3
C3
C3C
C3D
87.7 %
109
77.8 %
112
63.0 %
55
CD86
CD86+ cells (%)
CD86+cells (MFI)
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C3A
C3B
C3C
C3D

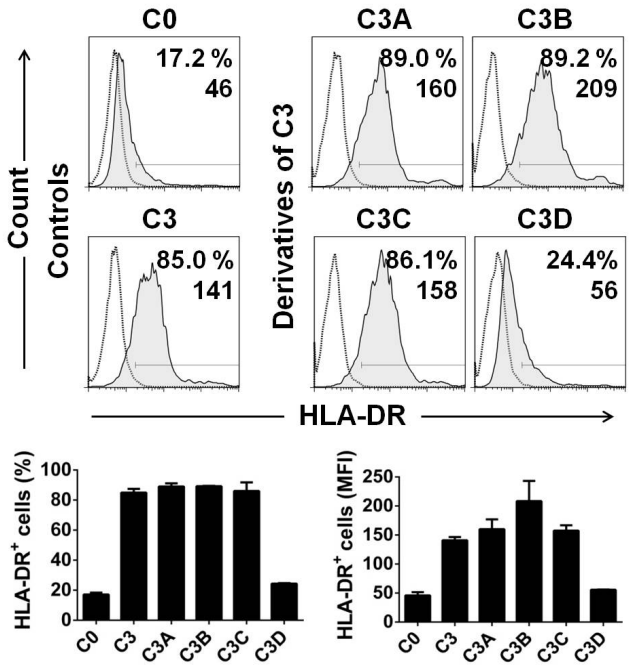


Figure 19. IFN-γ alone (C3C) is sufficient to upregulate expression of DC maturation markers. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Cells were immunostained for CD83, CD86, and HLA-DR and analysed by flow cytometry. Unshaded and grey histograms represent unstained control and indicated surface markers, respectively. Values in histograms are percentages of positive cells and MFI. Bar charts on the bottom depict mean percentages and the mean MFI ± standard deviations of data from two independent donors.

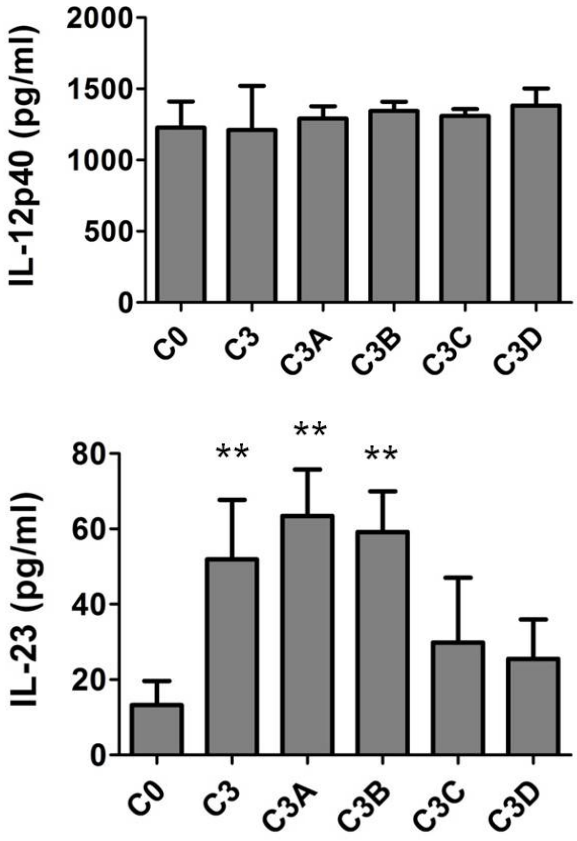


Figure 20. IFN-γ and R848 (C3B) are both required for IL-23 production. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Supernatants were then harvested and concentrations of IL-12p40 and IL-23 were determined by ELISA. Bar charts illustrate means ± standard deviations of triplicate measurements. ** $p<0.01$ using a two-tailed unpaired Student's *t*-test.

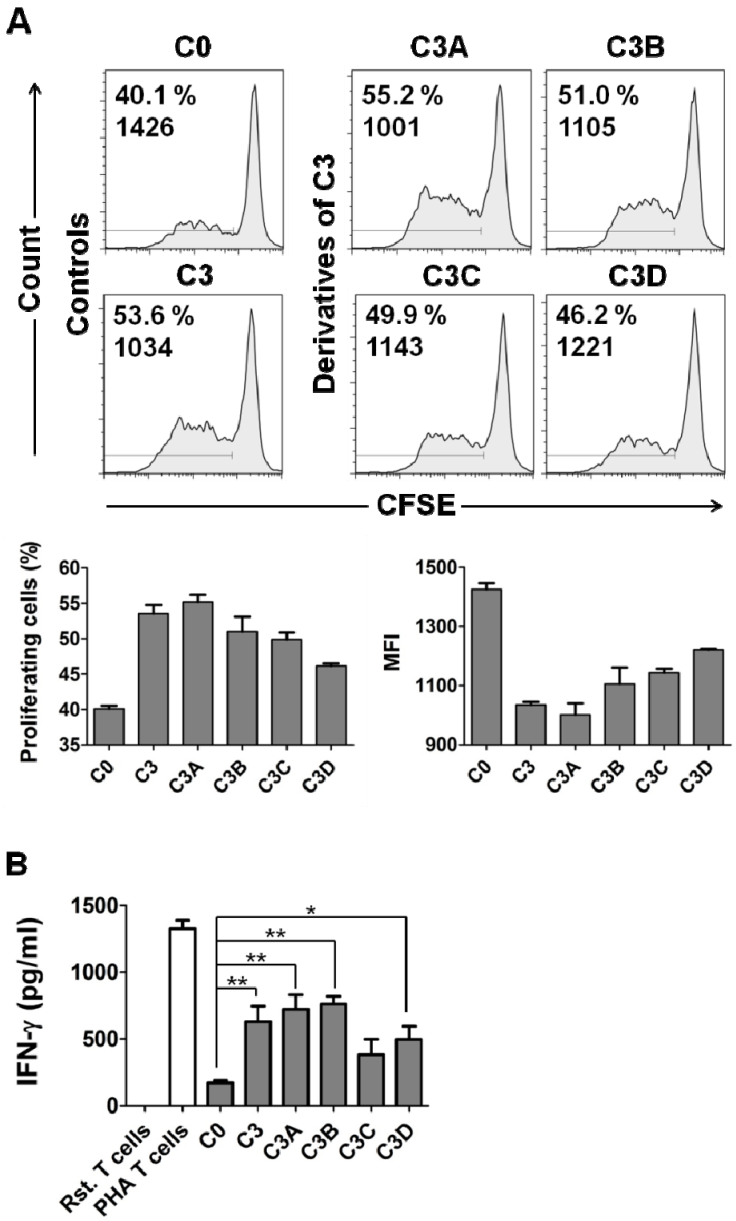


Figure 21. IFN-γ and R848 (C3B) are essential and sufficient for the optimal maturation of CD137L-DCs and subsequent T-cell activation. Monocytes were treated with immobilised CD137-Fc protein for 6 days and were further treated with the indicated maturation cocktails for 18 h. CD137L-DCs were then further co-cultured for 5 days with CFSE-stained allogeneic T cells at a ratio of 1:10. (A) Levels of T-cell proliferation were quantified by flow cytometry based on CFSE dilution. Values in the histograms and bar charts represent mean percentages of T cells undergoing CFSE-dilution and corresponding MFI of duplicate measurements. (B) Supernatants of day 5 co-cultures of mature CD137L-DCs with allogeneic T cells were harvested and levels of IFN-γ were determined by ELISA. Depicted are means ± standard deviations of triplicate measurements. *p<0.05; **p<0.01 using a two-tailed unpaired Student's t-test. This experiment has been performed twice with comparable results.

3.3.4. Optimisation of R848 and IFN-γ concentrations in maturation cocktail C3B

To facilitate the use of CD137L-DCs for future clinical settings, optimisation was performed to deduce the minimum concentration of R848 and IFN-γ required to mature CD137L-DCs without compromising on their resulting activity. To achieve this goal, R848 and IFN-γ were titrated in various combinations (table 6). Morphology and surface markers of treated CD137L-DCs were then documented.

Table 6: Optimising concentrations of IFN-γ and R848 for maturation of CD137L-DCs.

Cocktail	Components (concentration)	
	IFN-γ	R848
C0	-	-
C3B	250 ng/ml	1 μg/ml
C3B.1	50 ng/ml	1 μg/ml
C3B.2	10 ng/ml	1 μg/ml
C3B.3	250 ng/ml	200 ng/ml
C3B.4	250 ng/ml	40 ng/ml

Treatment with cocktail C3B.1, which contained a reduced IFN-γ concentration of 50 μg/ml, on CD137L-DCs produced near identical morphology as when C3B was used (figure 22). Cells were highly attached and had defined protrusions and spindles. Further reduction of IFN-γ concentration (10 μg/ml; C3B.2) abolished this effect and the CD137L-DCs were essentially morphologically similar to untreated CD137L-DCs. Decreased levels of R848 in both C3B.3 and C3B.4 yielded CD137L-DCs with attached morphology, however, there was clear reduction in spindle formation and cells were generally round and oval shaped. Likewise, only cocktail C3B.1 was able mature CD137L-DCs to the same degree as parental C3B, without compromising on the intensity of CD83, CD86 and HLA-DR

surface expression (figure 23). Therefore, maturation cocktail C3B.1 containing R848 at 1 μg/ml and IFN-γ at 50 ng/ml are the optimised concentrations which are sufficient to induce full maturation of CD137L-DCs. Henceforth, CD137L-DCs activated by this cocktail is termed 'mature CD137L-DCs'.

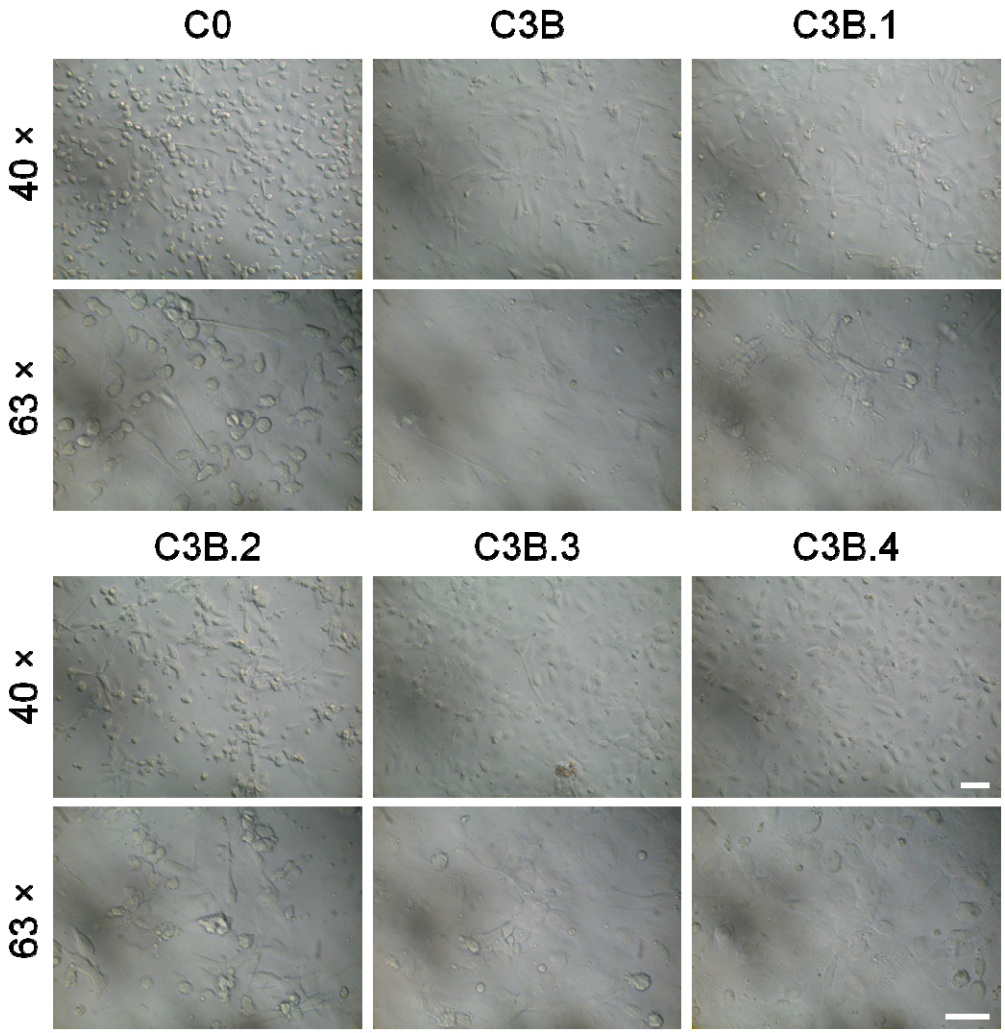


Figure 22. IFN-γ at 50 ng/ml plus R848 at 1 μg/ml (C3B.1) are required for morphological change of CD137L-DCs. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Photographs were taken on day 7 at magnification of 40 and 63 ×. Scale bar: 20 μm

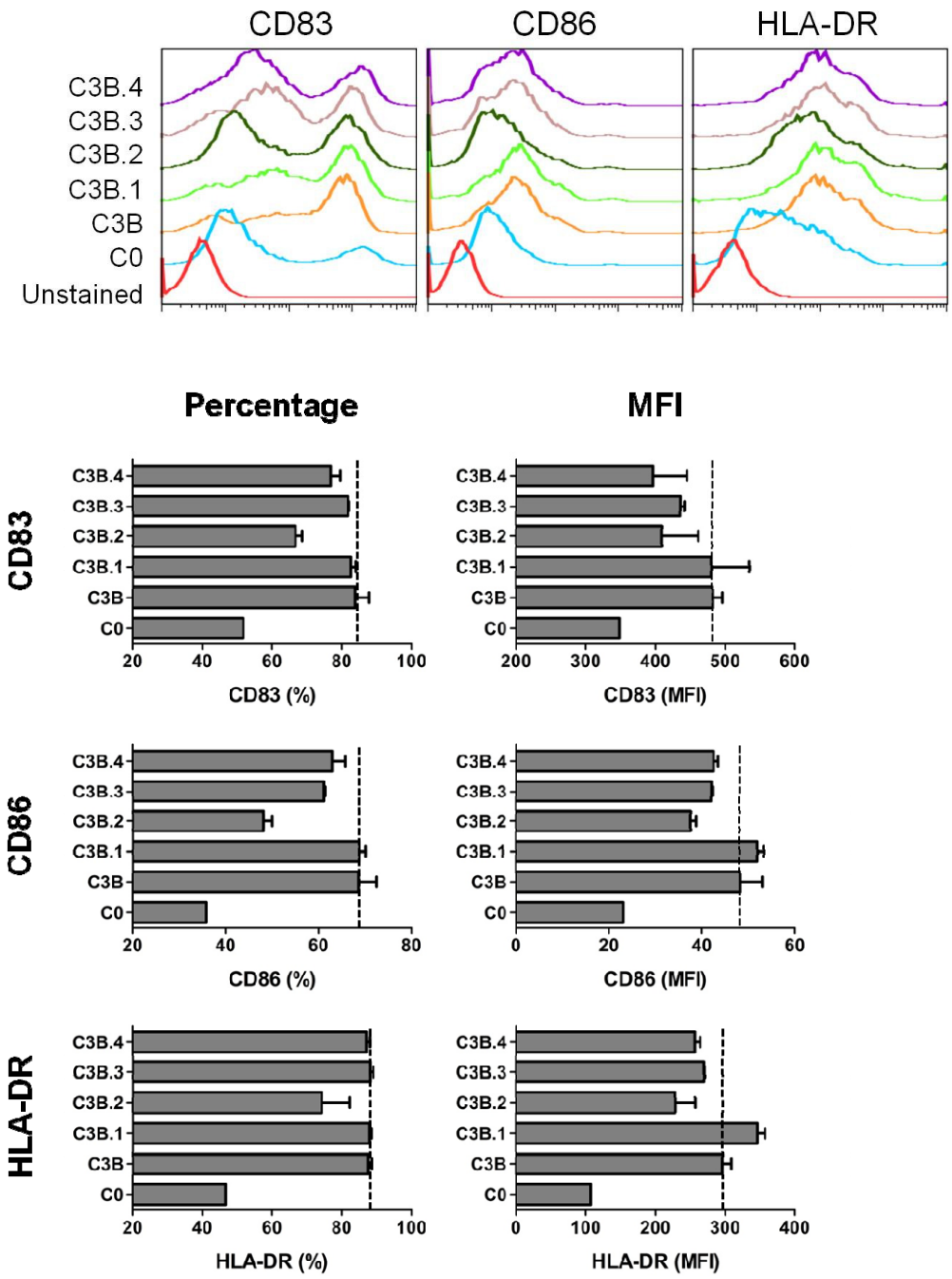


Figure 23. IFN-γ at 50 ng/ml plus R848 at 1 μg/ml (C3B.1) are required for optimal induction of DC maturation markers. Monocytes were treated with immobilised CD137-Fc protein for 6 days to generate CD137L-DCs and were further treated with the indicated maturation cocktails for 18 h. Cells were immunostained for CD83, CD86 and HLA-DR and analysed by flow cytometry. Bar charts on the bottom are corresponding mean percentages and the mean MFI ± standard deviations of duplicate samples. Vertical dotted line refers to mean percentage or MFI of indicated markers for parental C3B-treated CD137L-DCs. These data are representative of 2 independent experiments with comparable results.

3.4. CMV pp65 specific assay in an autologous system

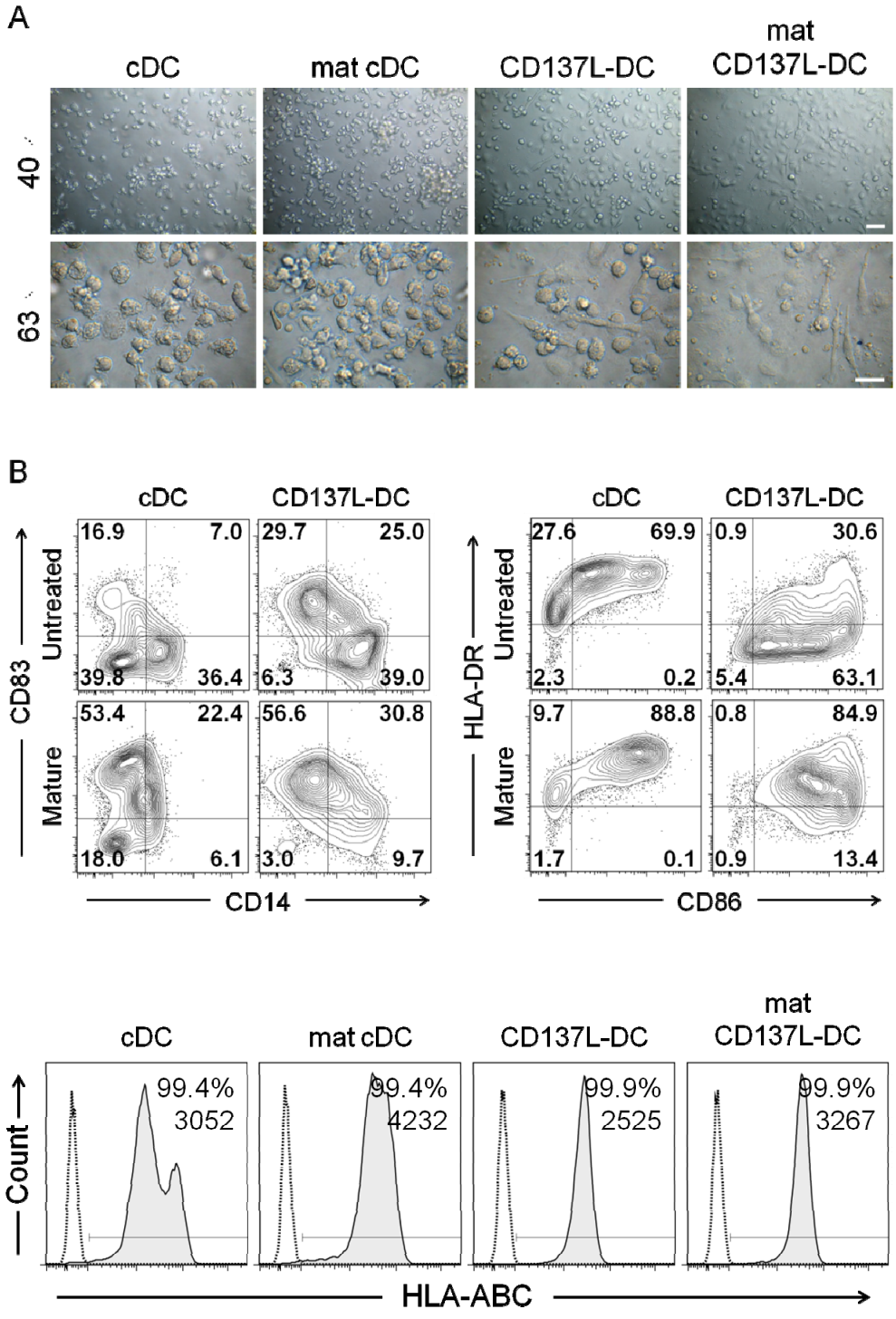
Having established an effective maturation cocktail for CD137L-DCs in an allogeneic system, the next question asked is whether these mature CD137L-DCs are capable - and potentially more effective than classical DCs - of initiating an autologous antigen-specific response. For this purpose, CMV pp65 recombinant protein and peptide pool were employed as a model antigen of interest and we generated pp65-specific T-cell lines from a $pp65^{+}$/HLA-$A2^{+}$ donor.

3.4.1. Generation of DCs

Classical DCs and CD137L-DCs were generated from autologous peripheral monocytes for 7 days and during the final 18 h, matured with a cocktail containing TNF, IL-1β, IL-6 and PGE2 (Jonuleit cocktail) or R848 plus IFN-γ (cocktail C3B.1 from 3.3.4), respectively. The Jonuleit cocktail is widely used in current clinical trials and is considered the gold standard for DC immunotherapy (Jonuleit et al., 1997). Therefore, classical DCs matured by the Jonuleit cocktail were used as a yardstick for the assessment of CD137L-DC activities. Classical DCs and CD137L-DCs differed morphologically even prior to maturation. Classical DCs were generally suspension cells with veil-like structures, and upon maturation developed many small dendrites while remaining non-adherent (figure 24A). CD137L-DCs on the other hand were mainly adherent and their maturation increased the frequency of more pronounced spindle protrusions and adherence, resulting in a distinct flatter morphology of the cells. Crucially, the expression of DC maturation markers CD83, CD86 and HLA-DR was upregulated in both mature classical DCs and mature CD137L-DCs with regards to their respective immature precursors

(figure 24B). Maturation also led to a reduction of the monocyte/macrophage marker CD14 and an increase in MHC class I (HLA-ABC) expression. These observations proved that the maturation cocktails for the respective DCs were indeed effective.

The growth factor GM-CSF and the cytokine IL-4 are essential for the differentiation and survival of classical DCs. However, CD137L-DCs are not supplemented with any exogeneous factors or cytokines during their differentiation from monocytes, and are therefore solely dependent on the effects of CD137L reverse signalling. To show that indeed signalling through CD137L is sufficient, the final numbers of DCs generated on day 7 were quantified. This is also essential as it is important to know how many DCs can be generated using a particular protocol. Using cell counting by microscopy and also by counting beads, it was deduced that there was no significant difference between the absolute number of DCs generated by either the classical protocol or by induction of CD137L signalling (figure 24C). In each case, 55 – 70 % of the monocytes had differentiated into DCs, and this was not affected by their subsequent maturation. Hence, CD137L signalling in monocytes can not only promote DC differentiation but also maintain their viability.


A
cDC
mat cDC
CD137L-DC
mat CD137L-DC
40
63
B
cDC
CD137L-DC
CD83
Untreated
Mature
16.9
7.0
39.8
36.4
29.7
25.0
6.3
39.0
53.4
22.4
18.0
6.1
56.6
30.8
3.0
9.7
CD14
HLA-DR
27.6
69.9
2.3
0.2
0.9
30.6
5.4
63.1
9.7
88.8
1.7
0.1
0.8
84.9
0.9
13.4
CD86
Count
99.4%
3052
99.4%
4232
99.9%
2525
99.9%
3267
HLA-ABC

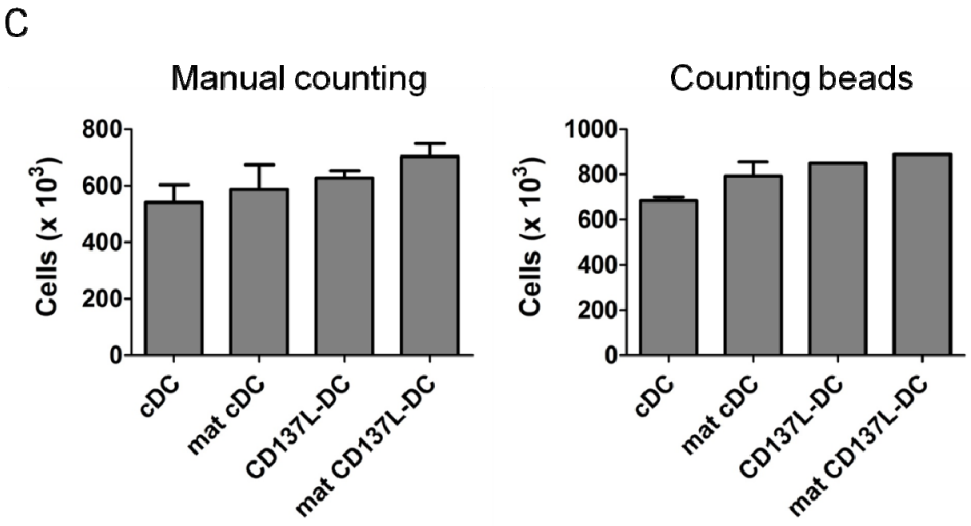


Figure 24. Generation and maturation of classical DCs and CD137L-DCs. Monocytes were treated with immobilised CD137-Fc protein (CD137L-DCs) for 7 days, or with 80 ng/mL GM-CSF plus 100 ng/mL IL-4 (classical DCs). For the final 18 h of culture, TNF, IL-1β, IL-6 and PGE2 were used to mature classical DCs, while IFN-γ (50 ng/ml) plus R848 (1 μg/ml) were used to mature CD137L-DCs. (A) Photographs were taken on day 7 at a magnification of 40 and 63 ×. Scale bar: 20 μm. (B) Cells were harvested and expression of CD14, CD83, CD86, HLA-DR, and HLA-ABC was determined by flow cytometry. These data are representative of at least 2-3 independent experiments with comparable results. (C) DC numbers were quantified by manual counting using microscopy or by counting beads using flow cytometry. Bar charts represent cell count per million cells seeded on day 0. Depicted are means ± standard deviations of triplicate countings. These data are representative of at least 2 - 3 independent experiments with comparable results.

3.4.2. Classical DCs are superior to CD137L-DCs when whole protein antigen is used

In this first approach, recombinant pp65 whole protein was utilised to comparatively assess the antigen-presenting capacity of classical DCs and CD137L-DCs. This would require the DCs to endocytose the antigen followed by its processing and subsequent expression of peptide antigen-MHC complex onto the cell surface. DCs were pulsed with pp65 protein at various concentrations, matured and then co-cultured with an autologous pp65-specific T-cell line (generated with pp65 protein) for 18 h before surface expression of the T-cell activating marker, CD137, was determined to measure activation.

To generate a pp65 T-cell line, PBMCs were pulsed with recombinant CMV pp65 protein. After 9-10 days, pulsed PBMCs were predominantly T cells based on their CD3 expression (figure 25). About 76 % of these T cells were $CD4^+$ while the rest were $CD8^+$.

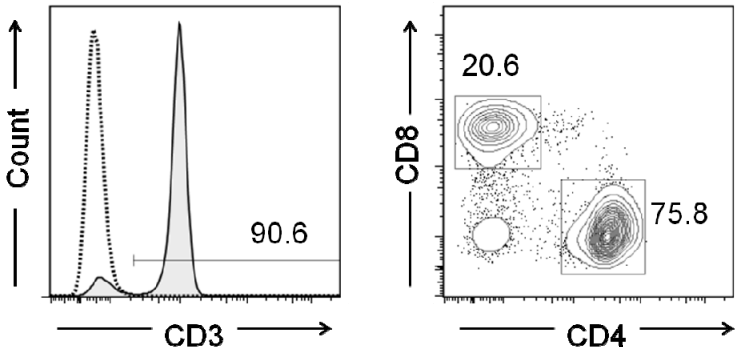
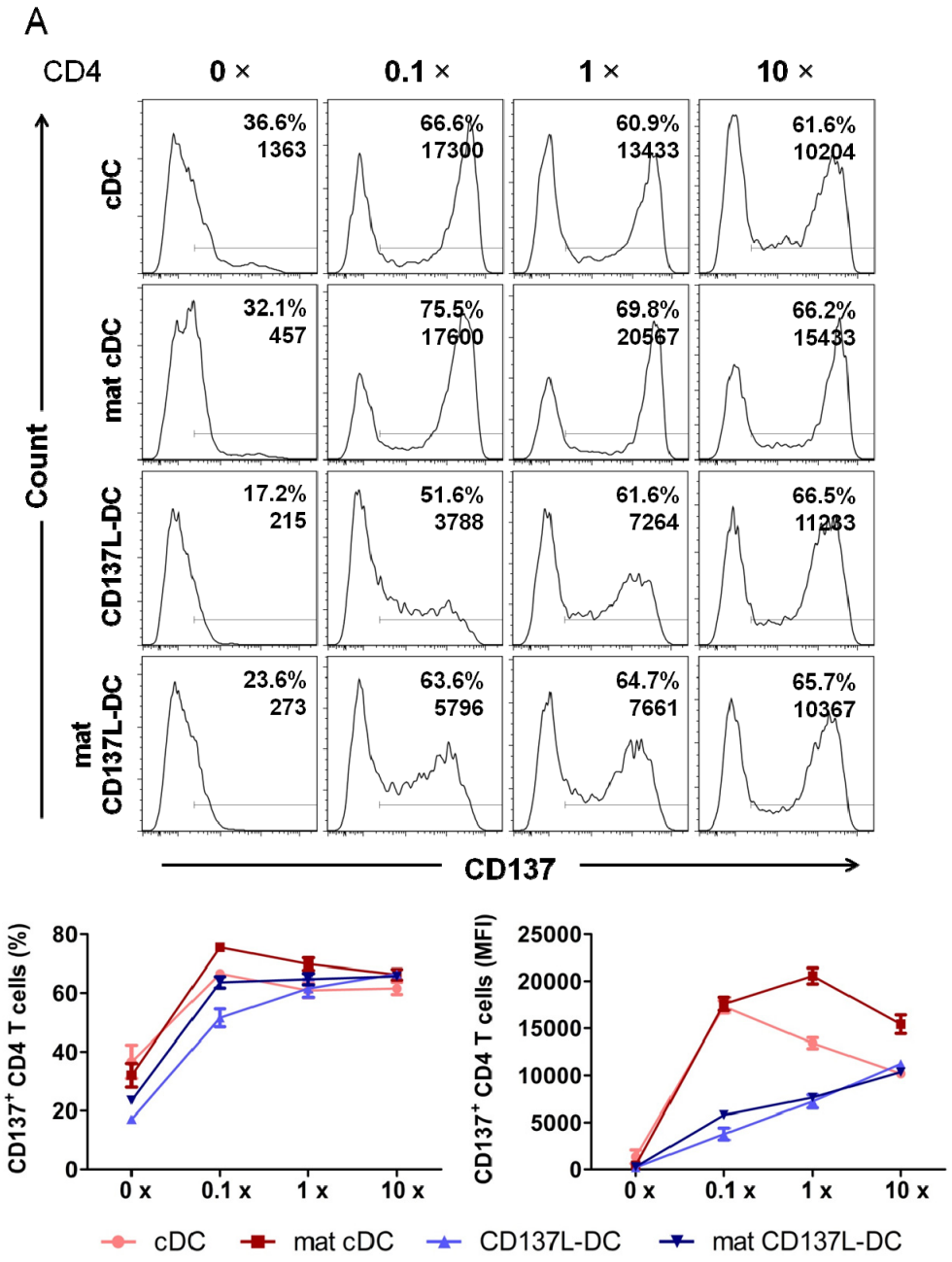


Figure 25. Phenotype of the pp65-specific T-cell line generated by recombinant pp65 protein. Donor PBMCs were stimulated with recombinant pp65 protein (10 μg/ml) for 9-10 days, with rIL-2 (10 U/ml) added after 24 h. Cells were immunostained for CD3, CD4, and CD8 and analysed by flow cytometry. In the left panel, unshaded and grey histograms represent unstained control and indicated surface marker, respectively. The right panel shows a dot plot of the population gated for $CD3^+$ cells. Values in the histogram and dot plot are percentages of positive cells from a single experiment. These data are representative of at least 2 independent experiments with comparable results.

Classical DCs are superior initiators of a pp65-specific $CD4^+$ and $CD8^+$ T-cell response. Although percentages of $CD4^+CD137^+$ T cells did not differ much amongst the various DC subsets, the MFI suggest that both immature and mature classical DCs are much more potent activators of $CD4^+$ T cells than CD137L-DCs even at the lowest antigen concentration (figure 26A). The $CD4^+$ T-cell activating ability of classical DCs reached a maximum at 0.1-1 × pp65 protein concentration and increasing antigen levels led to a reduction in T-cell activation. CD137L-DCs on the other hand, were able to increase, in an antigen dose-dependent manner, their capacity to activate $CD4^+$ T cells. However, both untreated and mature CD137L-DCs pulsed with even 10 × concentration of pp65 protein were not able to activate $CD4^+$ T cells more potently than mature classical DCs.

A similar response was noticed in the $CD8^+$ T-cell population. The lowest amount of pp65 antigen used (0.1 ×) was able to induce a maximum number of activated $CD8^+$ T cells by classical DCs while a further increase of pulsed-antigen was unable to supplement T-cell activation as the percentage of $CD137^+$ T cells had plateaued (figure 26B). CD137L-DCs were substantially less able to activate $CD8^+$ T cells although their capacity could be increased by pulsing with higher amounts of protein antigens. Altogether, these data shows that even though CD137L-DCs can perform cross-presentation, they are in this respect not as competent as classical DCs.


A
CD4
0 ×
0.1 ×
1 ×
10 ×
cDC
mat cDC
CD137L-DC
mat CD137L-DC
36.6%
1363
66.6%
17300
60.9%
13433
61.6%
10204
32.1%
457
75.5%
17600
69.8%
20567
66.2%
15433
17.2%
215
51.6%
3788
61.6%
7264
66.5%
11283
23.6%
273
63.6%
5796
64.7%
7661
65.7%
10367
Count
CD137
CD137+ CD4 T cells (%)
CD137+ CD4 T cells (MFI)
0 x
0.1 x
1 x
10 x
cDC
mat cDC
CD137L-DC
mat CD137L-DC

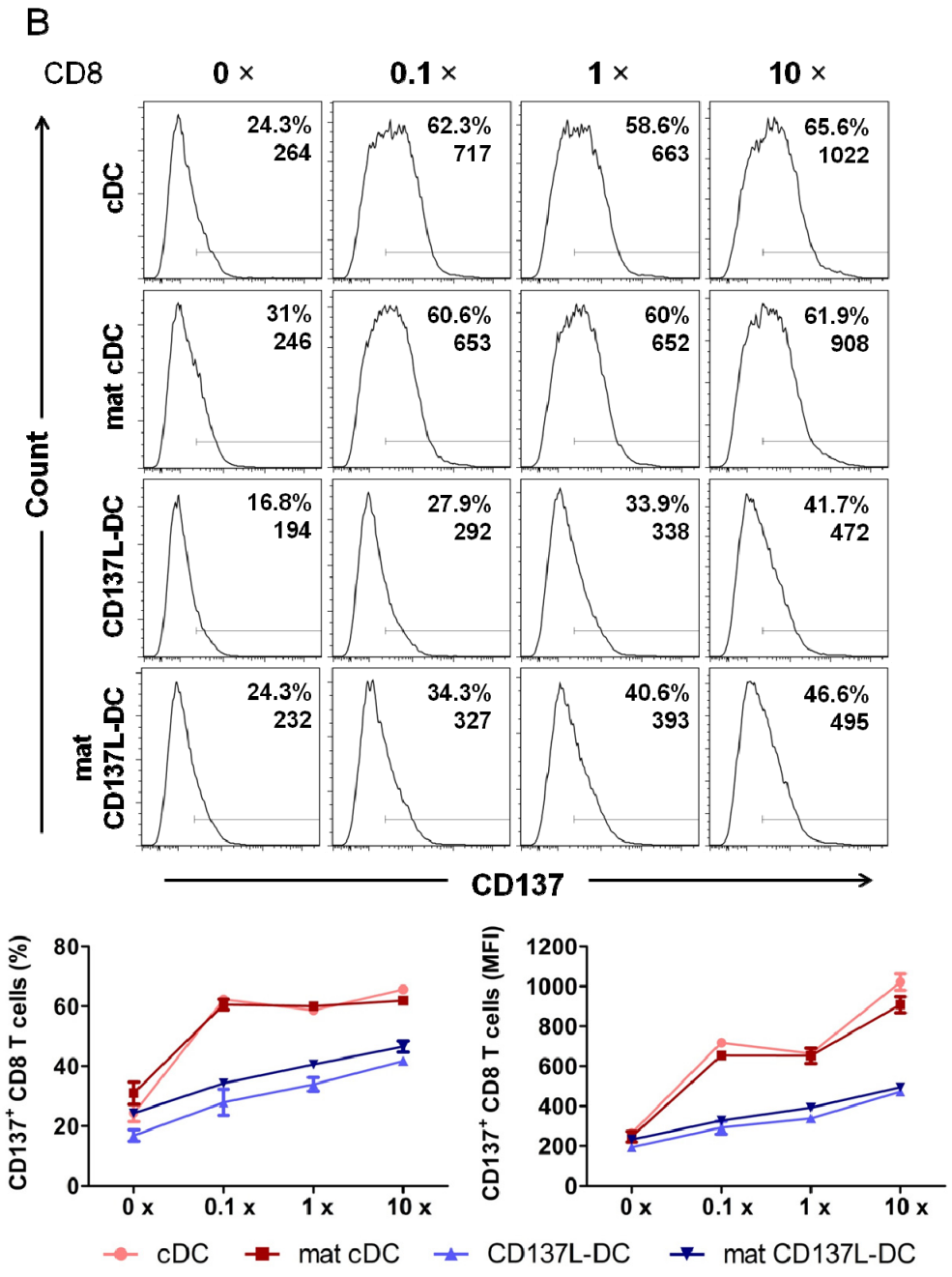


Figure 26. CD137L-DCs pulsed with whole pp65 proteins are weak T-cell activators. Day 7 DCs were pulsed with recombinant pp65 protein at the indicated concentrations (1× = 3.85 μg/ml) overnight with simultaneous maturation with either Jonuleit cocktail for classical DCs or R848 plus IFN-γ for CD137L-DCs. Day 9/10 pp65 T-cell line generated by pp65 proteins was harvested and co-cultured with protein-pulsed DCs at a ratio of 10:1 for 18 h. T cells were immunostained for the T-cell activation marker CD137, and co-stained for either CD4 (A) or CD8 (B) followed by analysis by flow cytometry. The line-graph represents the quantitative evaluation of all protein concentrations against various DC subsets. Depicted are means ± standard deviations of duplicate measurements of percentages and MFI.

3.4.3. Mature CD137L-DCs potently activate autologous, peptide pool-generated, antigen-specific T cells within 18 h

Besides using whole protein antigens, peptides are also commonly used in DC immunotherapy to stimulate an antigen-specific T-cell response. In this second approach, DCs were pulsed with pp65 peptides and were co-cultured with the autologous pp65-specific T-cell line for 18 h before expression of intracellular cytokines were measured to quantify T-cell activation.

To generate a pp65 T-cell line, PBMCs were pulsed with a CMV pp65 peptide pool. After 9-10 days, pulsed PBMCs were predominantly T cells based on their CD3 expression (figure 27). About 68-70 % of these T cells were $CD8^+$ while the rest were $CD4^+$.

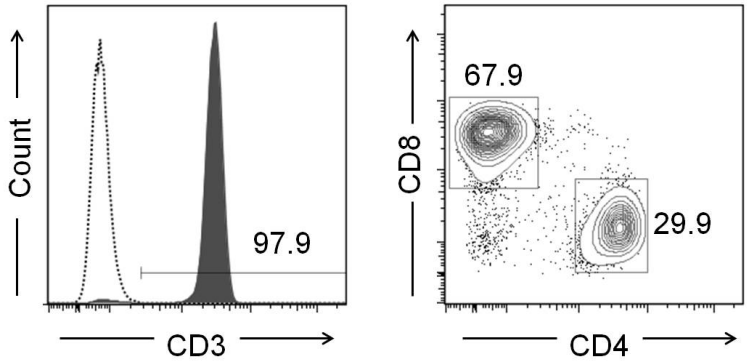


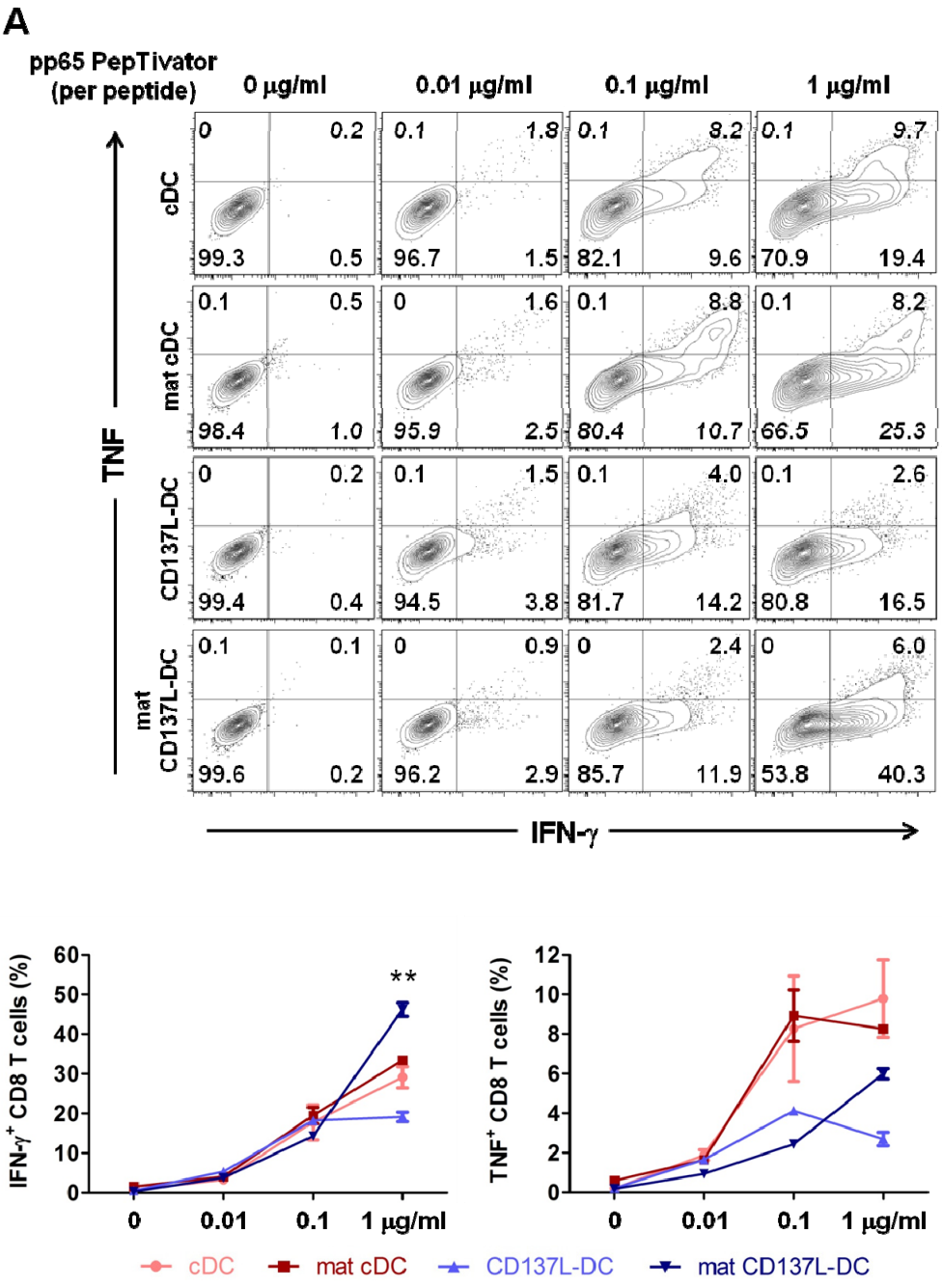
Figure 27. Phenotype of the pp65-specific T-cell line generated by the pp65 peptide pool. Donor PBMCs were stimulated with PepTivator pp65 spanning peptide pool (0.1 μg/ml per peptide) for 9-10 days, with rIL-2 (10 U/ml) added after 24 h. Cells were immunostained for CD3, CD4, and CD8 and analysed by flow cytometry. In the left panel, unshaded and grey histograms represent unstained control and indicated surface marker, respectively. The right panel shows a dot plot of the population gated for $CD3^+$ cells. Values in the histogram and dot plot are percentages of positive cells from a single experiment. These data are representative of at least 3 independent experiments with comparable results.

In the absence of pp65 peptides, T cells remained unactivated and did not produce any of the cytokines tested (figure 28). The different types of DCs were equally able to activate $CD8^+$ T cells in an antigen- and dose-dependent manner, however at 1 μg/ml per peptide concentration, T-cell activation was significantly potentiated by mature CD137L-DCs as denoted by the IFN-γ expression (46.2 % of T cells), (figure 28A). Untreated CD137L-DCs were least able to activate pp65-specific T cells at this high antigen concentration (19.2 % of T cells). About 29-33 % of $CD8^+$ T cells were activated by immature/mature classical DCs. Besides IFN-γ, TNF is also produced by pp65-specific $CD8^+$ T cells (Shin et al., 2013). Despite the ability of mature CD137L-DCs to induce a high percentage of IFN-γ-producing $CD8^+$ T cells, they were unable to initiate potent TNF production by these T cells (5.9 % of T cells). On the other side, both immature and mature classical DCs were much more potent in promoting TNF production (9.8 % and 8.3 % of T cells, respectively). Untreated CD137L-DCs were only able to promote a maximum TNF production in 4.1 % of $CD8^+$ T cells. Regardless of the capacity to induce TNF production in T cells, mature CD137L-DCs were potent activators of $CD8^+$ T cells as IFN-γ production is considered a key cytokine that signifies functional activation of cytotoxic T cells.

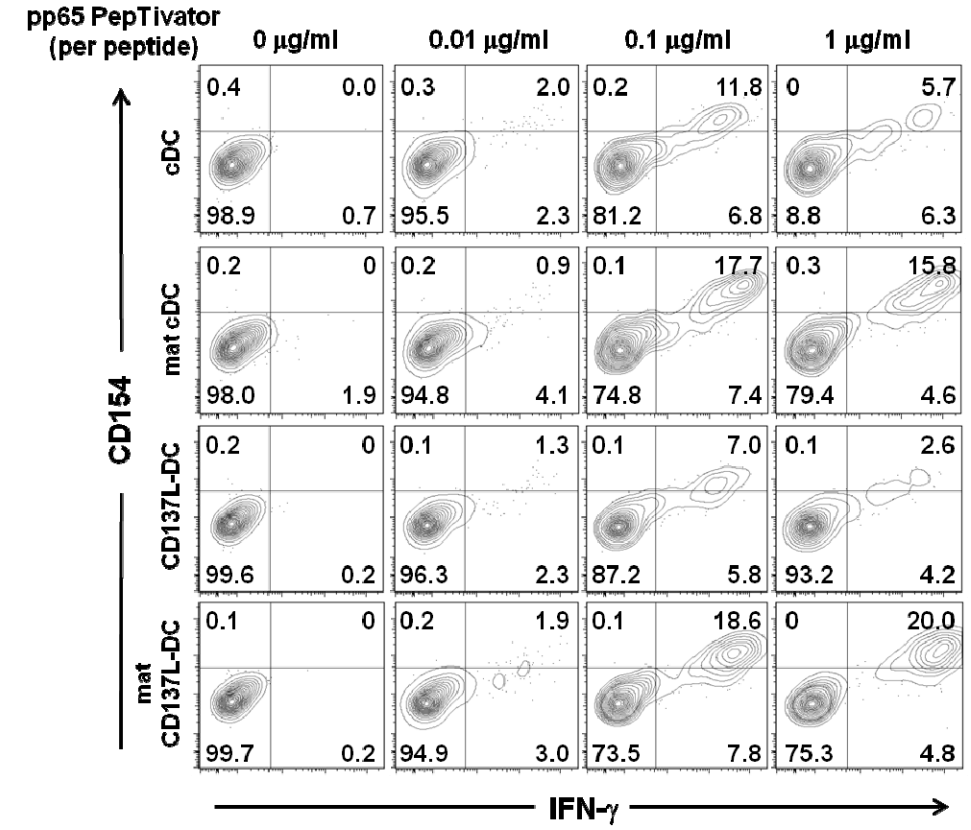
A similar pattern was observed for the priming of $CD4^+$ T cells by the different DC populations (figure 28B). $CD4^+$ T cells remained inactive in the absence of pp65 peptides and only a small population was minimally activated in the presence of pp65 peptide at a concentration of 0.01 μg/ml. Maximal T-cell activation was achieved at a peptide concentration of 0.1 μg/ml while a further increase in loaded peptides (1 μg/ml) led to a slight reduction in T-cell activation and this trend was observed in all DC types. More importantly, the mature form of classical DCs and CD137L-DCs were more potent activators

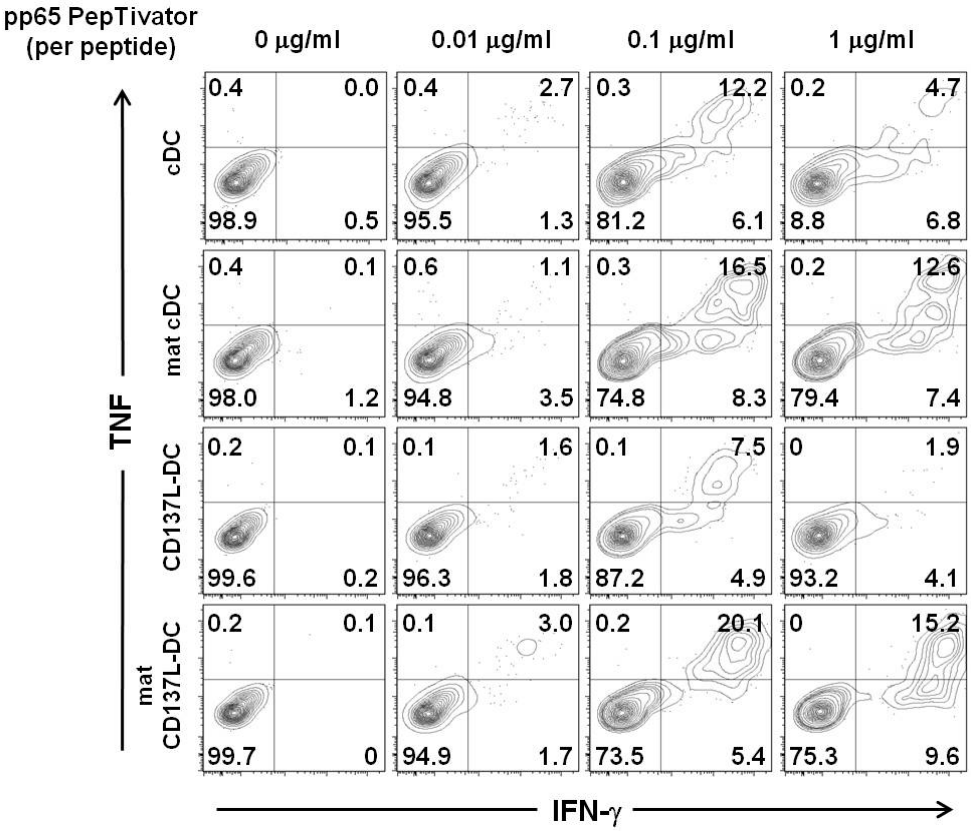
of $CD4^+$ T cells than their immature counterpart. At 0.1 μg/ml per peptide concentration, 25-26 % of T cells produced IFN-γ when co-cultured with the mature form the DCs while only 18.3 % and 12.4 % were IFN-γ^+ when restimulated by immature classical DCs and untreated CD137L-DCs, respectively. Unlike their effects on $CD8^+$ T cells, mature CD137L-DCs were able to activate $CD4^+$ T cells to not only produce IFN-γ but also TNF. In fact, mature CD137L-DCs and mature classical DCs were the strongest activators of TNF-producing $CD4^+$ T cells. A similar pattern was also observed for the expression of CD154; a $CD4^+$ activation marker. Untreated CD137L-DCs were the least effective APCs in instilling a $CD4^+$ T-cell response.

Collated data from four independent experiments validated that mature CD137L-DCs are indeed more potent activators of both antigen-specific $CD8^+$ and $CD4^+$ T cells as compared to the other DC types (figure 29).


A
pp65 PepTivator (per peptide)
0 µg/ml
0.01 µg/ml
0.1 µg/ml
1 µg/ml
cDC
mat cDC
CD137L-DC
mat CD137L-DC
TNF
IFN-γ
0 0.2 99.3 0.5
0.1 1.8 96.7 1.5
0.1 8.2 82.1 9.6
0.1 9.7 70.9 19.4
0.1 0.5 98.4 1.0
0 1.6 95.9 2.5
0.1 8.8 80.4 10.7
0.1 8.2 66.5 25.3
0 0.2 99.4 0.4
0.1 1.5 94.5 3.8
0.1 4.0 81.7 14.2
0.1 2.6 80.8 16.5
0.1 0.1 99.6 0.2
0 0.9 96.2 2.9
0 2.4 85.7 11.9
0 6.0 53.8 40.3
IFN-γ+ CD8 T cells (%)
60
50
40
30
20
10
0
**
0
0.01
0.1
1 µg/ml
TNF+ CD8 T cells (%)
12
10
8
6
4
2
0
0
0.01
0.1
1 µg/ml
cDC
mat cDC
CD137L-DC
mat CD137L-DC

B


pp65 PepTivator
(per peptide)
0 µg/ml
0.01 µg/ml
0.1 µg/ml
1 µg/ml
cDC
mat cDC
CD137L-DC
mat CD137L-DC
CD154
IFN-γ
0.4 0.0 98.9 0.7
0.3 2.0 95.5 2.3
0.2 11.8 81.2 6.8
0 5.7 8.8 6.3
0.2 0 98.0 1.9
0.2 0.9 94.8 4.1
0.1 17.7 74.8 7.4
0.3 15.8 79.4 4.6
0.2 0 99.6 0.2
0.1 1.3 96.3 2.3
0.1 7.0 87.2 5.8
0.1 2.6 93.2 4.2
0.1 0 99.7 0.2
0.2 1.9 94.9 3.0
0.1 18.6 73.5 7.8
0 20.0 75.3 4.8


pp65 PepTivator
(per peptide)
0 µg/ml
0.01 µg/ml
0.1 µg/ml
1 µg/ml
cDC
mat cDC
CD137L-DC
mat CD137L-DC
TNF
IFN-γ
0.4 0.0 98.9 0.5
0.4 2.7 95.5 1.3
0.3 12.2 81.2 6.1
0.2 4.7 8.8 6.8
0.4 0.1 98.0 1.2
0.6 1.1 94.8 3.5
0.3 16.5 74.8 8.3
0.2 12.6 79.4 7.4
0.2 0.1 99.6 0.2
0.1 1.6 96.3 1.8
0.1 7.5 87.2 4.9
0 1.9 93.2 4.1
0.2 0.1 99.7 0
0.1 3.0 94.9 1.7
0.2 20.1 73.5 5.4
0 15.2 75.3 9.6

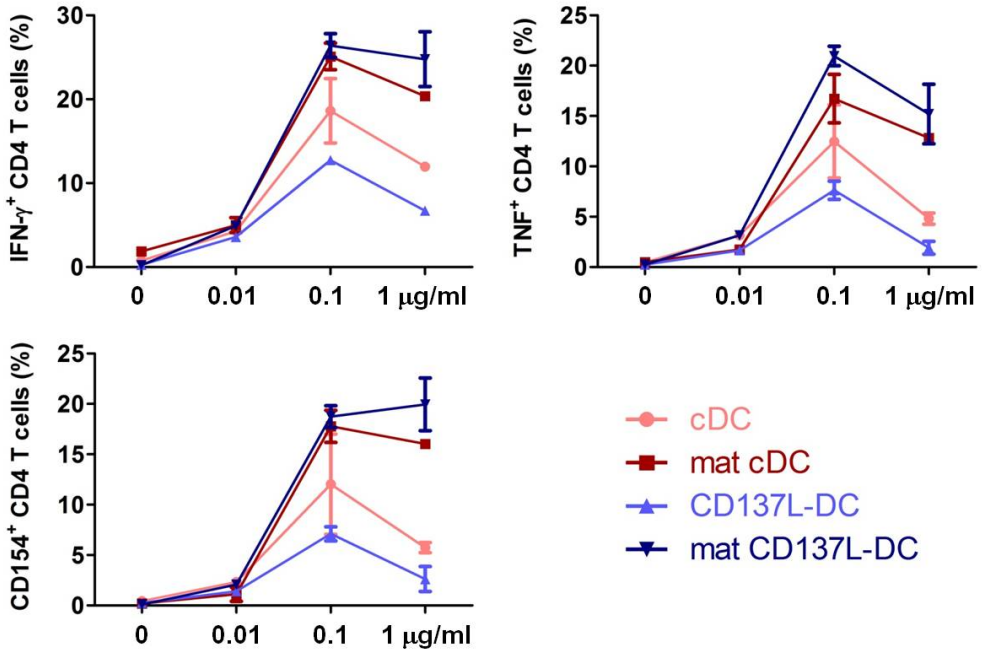


Figure 28. Mature CD137L-DCs pulsed with a pp65 peptide pool are potent T-cell activators. Day 7 DCs were left untreated or matured with either the Jonuleit cocktail for classical DCs, or R848 plus IFN-γ for CD137L-DCs for 18 h. Upon maturation, DCs were pulsed with the pp65 peptide pool at 0.01 μg/ml, 0.1 μg/ml or 1 μg/ml of each peptide or no peptides as a control for 2 h at 37ºC. Day 9/10 pp65 T-cell line generated by pp65 peptide pool was harvested and co-cultured with protein-pulsed DCs at a ratio of 10:1 in the presence of 2 μg/ml Brefeldin A for a further 18 h. T cells were immunostained intracellularly for IFN-γ, TNF, and CD154 (only for $CD4^+$ T cells) and co-stained for either CD8 (A) or CD4(B) followed by analysis by flow cytometry. Line-graphs represent the quantitative evaluation of all peptide concentrations against the various DC subsets. Depicted are means ± standard deviations of percentages of duplicate measurements. $^{**}p<0.01$ using a two-tailed unpaired Student's *t*-test.

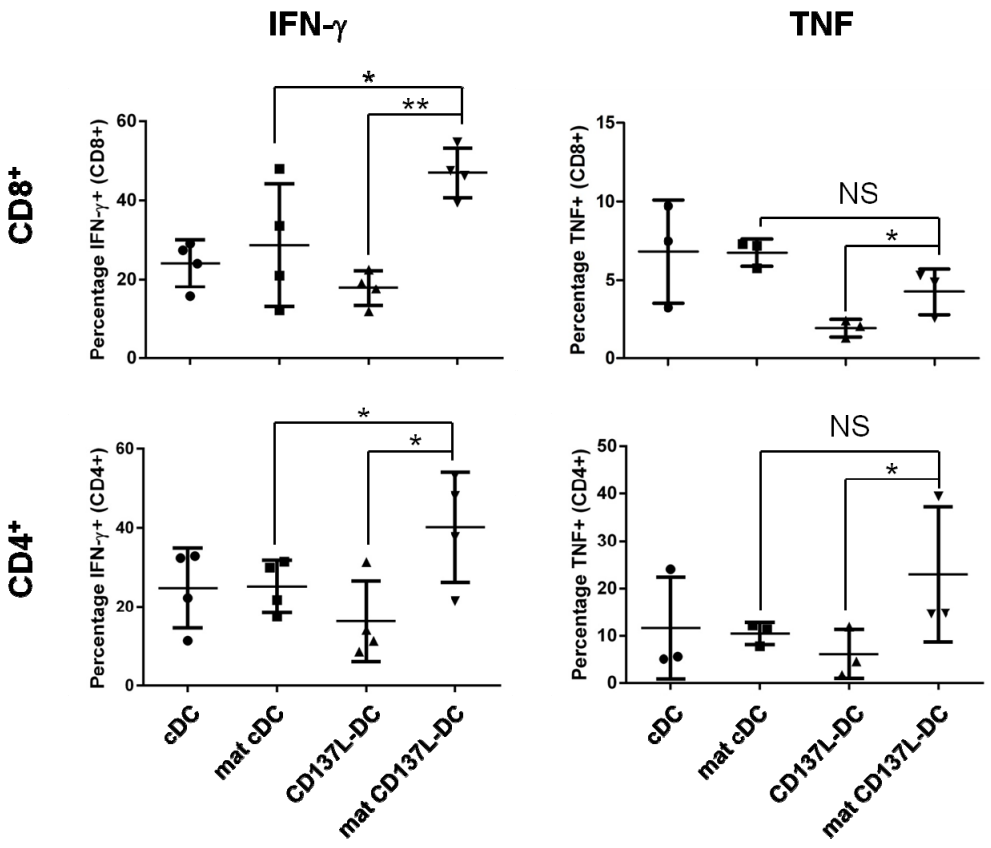


Figure 29. Mature CD137L-DCs potently induce IFN-γ but not TNF production in both CD8$^+$ and CD4$^+$ T cells. Scatter plot depicting collated data of IFN-γ-secreting or TNF-secreting CD8$^+$ or CD4$^+$ pp65-specific T cells from 3-4 independent experiments. Day 7 DCs were left untreated or matured with either the Jonuleit cocktail for classical DCs, or R848 plus IFN-γ for CD137L-DCs for 18 h. Upon maturation, DCs were pulsed with the pp65 peptide pool at 1 μg/ml of each peptide for 2 h at 37ºC. Day 9/10 pp65 T-cell line generated by pp65 peptide pool was harvested and co-cultured with protein-pulsed DCs at a ratio of 10:1 in the presence of 2 μg/ml Brefeldin A for a further 18 h. T cells were immunostained intracellularly for IFN-γ and TNF, and co-stained for either CD8 or CD4 followed by analysis by flow cytometry. Depicted are percentage and MFI means ± standard deviation. **p*<0.05; ***p*<0.01 using a two-tailed unpaired Student's *t*-test. NS = not significant.

3.4.4. Both untreated and mature CD137L-DCs are able to potently activate autologous, peptide pool-generated, antigen-specific T cells in 5 days

Having demonstrated that mature CD137L-DCs are superior antigen-specific T-cell activators, the next logical step was to characterise the T cells restimulated by the various DCs. Rather than investigating the T cells after a short reactivation period, T cells were co-culture with the various pp65 peptides-pulsed DCs for 5 days prior to further analysis. This mimics the physiological situation where DC interaction with T cells is dynamic and occurs in three distinct phases over a period of 48 h or more (Mempel et al., 2004).

3.4.4.1. Morphology of DC-T-cell co-culture

Photographs of the co-culture were acquired on day 5, and in the absence of pp65 peptides, a small number of T-cell clumps were observed in all co-culture conditions (figure 30). These clumps could be due to the presence of residual activated T cells and not due to their restimulation by the DCs. T cells co-cultured with antigen pulsed untreated or mature CD137L-DCs formed not only more aggregates but ones that were also larger in size and grew in clonal suspension in an apparent homotypic aggregate. When observed at a higher magnification, co-cultures with either pulsed immature or mature classical DCs were able to drive T cells to form larger aggregates than in their respective unpulsed condition. Nevertheless, T-cell aggregates were markedly smaller than those cultured with CD137L-DCs implying that CD137L-DCs, whether untreated or matured, are better T-cell restimulators over a longer period.

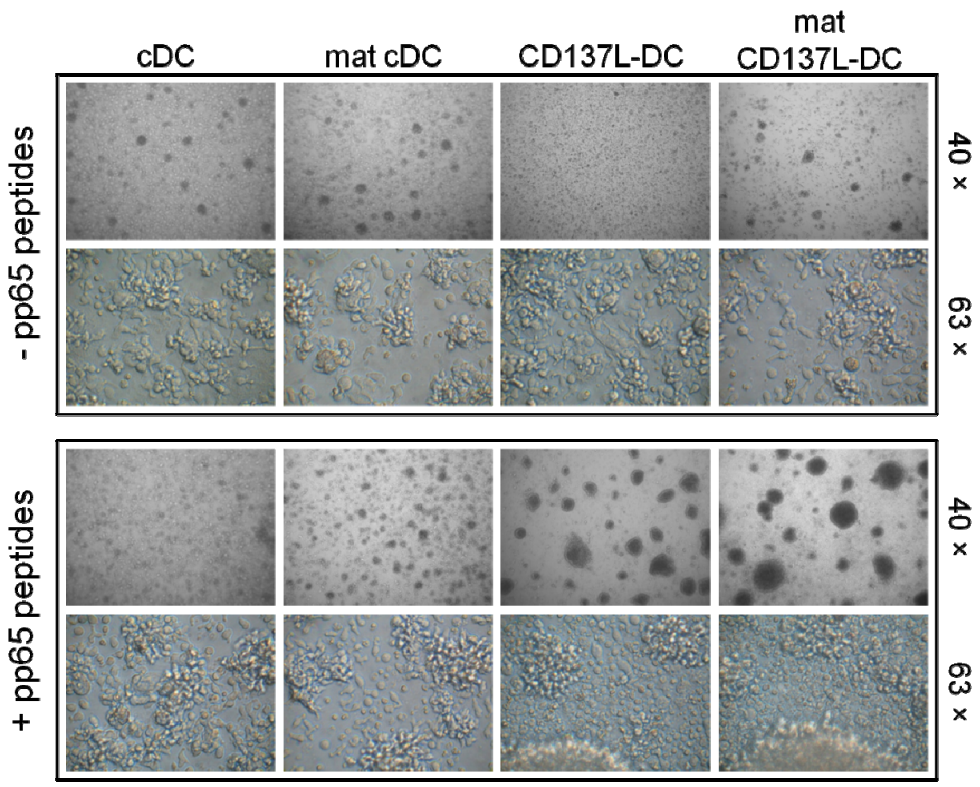


Figure 30. pp65 T cells cultured with CD137L-DCs form large aggregates. DCs were either used unpulsed or pulsed with pp65 peptide pool (at 0.1 μg/ml per peptide) and co-cultured with day 9/10 pp65-specific T cells at a ratio of 1:10. Photographs were taken on day 5 at magnifications of 40 and 63 ×.

3.4.4.2. Cytokine production and T-cell phenotype

DCs can not only present antigens but also determine the outcome of an immune response by inducing T-cell activation and polarisation. Hence, T-cell function is more often than not directly influenced by the stimulating DCs, and their cytokine production is a good phenotypic indicator of the T cells generated.

Pro-inflammatory T cells can be divided into 3 main subsets, namely Th1, Th2 and Th17, and are distinguishable by the type of cytokines they produce. Th1 cells generally secrete IFN-γ and TNF, Th2 cells secrete IL-4 and IL-13 while Th17 cells produce IL-17 (Kadowaki, 2007; Kurts, 2008). Anti-inflammatory

Tregs can be characterised by the production of IL-10. Activated CMV pp65 T cells predominantly produce the inflammatory cytokines IFN-γ and TNF and therefore skew towards a Th1 response (Kiecker et al., 2004; Shin et al., 2013). Indeed, in all DC conditions, day 5 reactivated T cells were able to secrete IFN-γ and TNF in response to pp65 peptide presentation. This is in line with the previous data in section 3.4.3 which shows that IFN-γ was produced by pp65-specific T cells within a short time span especially when there were reactivated by mature CD137L-DCs (figure 29). Nonetheless, longer periods of restimulation by either untreated or mature CD137L-DCs rendered higher overall IFN-γ and TNF production by T cells compared to those restimulated by the classical DCs. Untreated CD137L-DCs induced 2 times more IFN-γ (14.6 ± 0.6 ng/ml vs 7.2 ± 0.2 ng/ml) and 2.5 times more TNF (301 ± 8 pg/ml vs 120 ± 2 pg/ml) compared to mature classical DCs (figure 31). Mature CD137L-DCs were most effective and T cells restimulated by them produced by far the highest amount of IFN-γ (25.7 ± 1 ng/ml) and TNF (324 ± 6 pg/ml). Collectively, despite the inefficiency of CD137L-DCs to strongly activate pp65 T cells within 18 h (figure 29), it is clear that a longer period of T-cell-DC interaction is required for untreated CD137L-DCs to exert their potent T-cell activating function.

CD137L-DCs have been shown to activate not only Th1 but also Th2 and Th17 subsets of $CD4^+$ T cells (Kwajah and Schwarz, 2010). Thus, production of IL-13 and IL-17 were also analysed. Basal levels of IL-13 were produced (< 3 ng/ml) in the absence of stimulation which were heightened upon restimulation with peptide-pulsed DCs (figure 31). Co-culture of T cells with untreated CD137L-DCs was more effective than classical DCs in elevating production of IL-13 (4.4 ± 0.1 ng/ml vs 2.7 ± 0.1 ng/ml) and even more so by mature CD137L-DCs (5.4 ± 0.1 ng/ml). Although CD137L-DCs are capable of

polarising $CD4^+$ T cells towards a Th2 phenotype, this is surprising, in the context of CMV, as pp65 is not known to induce IL-13 production in T cells. Instead, CMV infection induces an inflammatory response with a distinct type 1 cytokine signature (van de Berg et al., 2010). The other cytokines tested, IL-17 and IL-10, were not detected in the co-culture supernatants suggesting that Th17 cells and Tregs are not activated by CMV infection (data not shown).

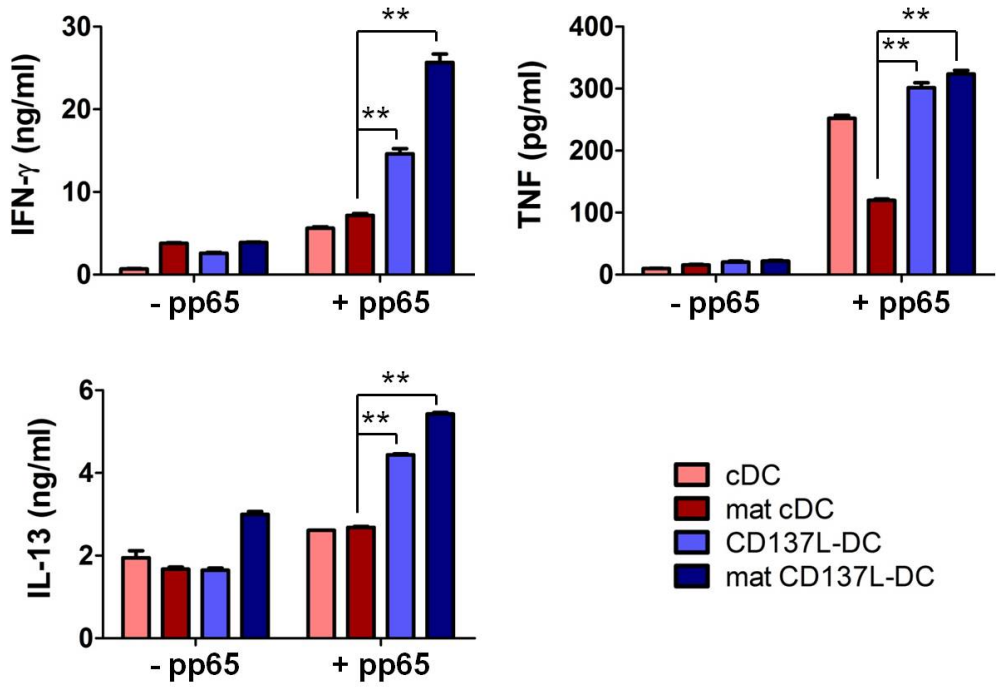


Figure 31. T cells stimulated by CD137L-DCs for 5 days induce high Th1 and Th2 cytokine secretion. DCs were pulsed with a pp65 peptide pool or were used unpulsed. T cells specific for pp65 were co-cultured with the peptide-pulsed DCs at a ratio of 10:1. IL-2, IL-7 and IL-15 were added on day 3 of restimulation. Concentrations of IFN-γ, TNF and IL-13 in day 5 supernatants were determined by ELISA. Depicted are means ± standard deviations of percentages of triplicate measurements. These data are representative of at least two independent experiments with comparable results. ** $p<0.01$ using a two-tailed unpaired Student's *t*-test.

IL-13, TNF and IFN-γ are cytokines that are predominantly synthesised by $CD4^+$ T cells, although the latter is also produced by cytotoxic $CD8^+$ T cells. Since CD137L-DCs, especially in the mature form, were able to strongly initiate the production of these cytokines by T cells, we speculated that there may be a shift in the CD8/CD4 T-cell ratio towards a $CD4^+$ T-cell bias when pp65 T cells are primed by CD137L-DCs. Day 5 co-cultures attained were predominantly T cells as more than 96 % were $CD3^+$ (figure 32A). More notably, pp65-specific T cells restimulated with all types of DCs, with the exception of mature CD137L-DCs, had maintained or slightly reduced (collated data from 3 independent experiments) their CD4/CD8 population phenotype as compared to their initial phenotype before reactivation (figure 27 and 32B, respectively). On the other hand, mature CD137L-DCs shifted the T-cell ratio towards a CD4 phenotype as evidenced by the increased percentage of $CD4^+$ T cells (29.9 % vs 37.1 %) and the simultaneous percentage reduction of the $CD8^+$ T cells (67.9 % vs 60.7 %) with respect to their phenotype prior to restimulation (figure 32A). Such a shift towards a CD4 phenotype upon restimulation by mature CD137L-DCs was validated from collated data of three independent experiments (figure 32B).

In this regard, we suggest that untreated CD137L-DCs are indeed more potent activators of $CD4^+$ T cells than classical DCs as there was no significant shift in the CD8/CD4 ratio when restimulated by either DCs, and therefore the stark increase in IL-13, TNF and IFN-γ is likely contributed solely to the robust function of CD137L-DCs to drive a strong $CD4^+$ T-cell response (and perhaps also $CD8^+$ T-cell response as IFN-γ is also highly produced by these cells). On the other hand, it is likely that mature CD137L-DCs have a similar potency as untreated CD137L-DCs in inducing a $CD4^+$ T-cell response, however, the shift of the ratio towards the $CD4^+$ T-cell population

would mean that there are more $CD4^+$ T cells that can contribute to an even higher production of IL-13, TNF and IFN-γ.

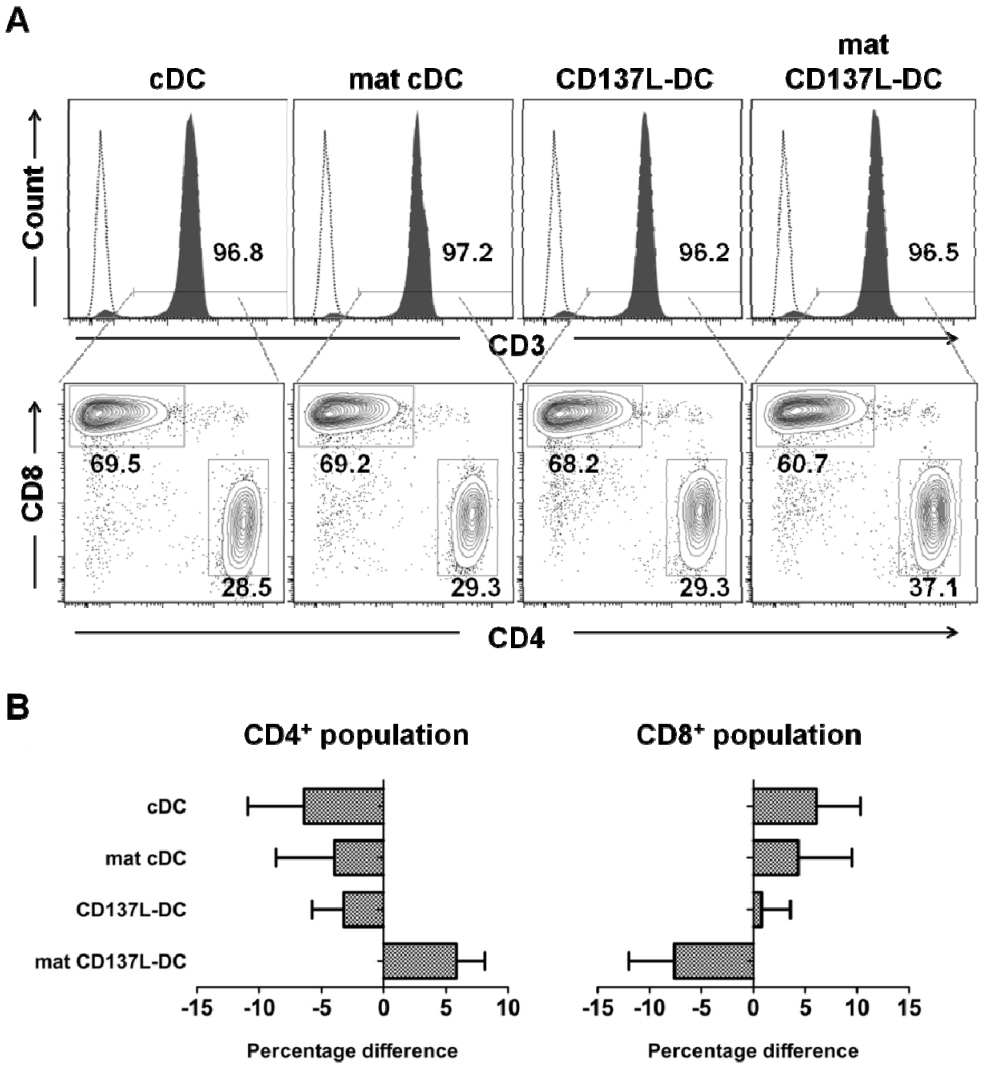


Figure 32. Mature CD137L-DCs alter the CD4/CD8 T-cell ratio towards a $CD4^+$ T-cell bias. DCs were pulsed with a pp65 peptide pool or were used unpulsed. T cells specific for pp65 were co-cultured with the peptide-pulsed DCs at a ratio of 10:1. IL-2, IL-7 and IL-15 were added on day 3 of restimulation. Cells were immunostained for CD3, CD4 and CD8, and were analysed by flow cytometry. (A) Bottom panel shows cells gated on the $CD3^+$ population. Values in histograms depict percentages of a single experiment. (B) Bar chart depicts percentage difference of $CD4^+$ T cells (left panel) and $CD8^+$ T cells (right panel) with respect to the initial pre-restimulated pp65 T-cell line population. Data was collated from 3 independent experiments.

3.4.5. Both untreated and mature CD137L-DCs induce superior killing activity in antigen-specific autologous T cells

Both untreated and mature forms of CD137L-DCs are able to prime pp65-specific T cells and this leads to a large production of TNF and IFN-γ in a 5 day restimulatory condition. Additionally, mature CD137L-DCs were able to potently activate antigen-specific $CD8^+$ T cells within 18 h as evidenced by the IFN-γ production. Altogether, this profile suggests that mature CD137L-DCs are able to initiate a powerful Th1 and CTL response towards specific antigens. To prove this hypothesis, the killing activity of T cells restimulated by the different DCs was determined by assessing their potency to lyse pp65 peptides-pulsed target cells in a cytotoxicity assay.

To ensure that target cell lysis is due to recognition of peptide-antigens and not HLA mismatch, it was crucial that the donor and target cells were HLA-matched. Haplotyping of donor monocytes and the target lymphoblastoid B cell line (clone CM371) confirmed that they were indeed HLA-matched as both were HLA-$A2^+$ (figure 33A).

Dose-dependent killing of target cells was observed at increasing effector/target (E:T) ratios in all conditions (figure 33B). Notably, maturation of classical DCs or CD137L-DCs had no or little effect, respectively, on their ability to induce cytotoxic activity in the T cells. In the absence of pp65 peptides, immature or mature classical DCs restimulated T cells had no cytotoxic activity while T cells restimulated by CD137L-DCs, with or without maturation, killed 4 – 20% of the target cells. Regardless of the restimulatory DC, pulsing of target cells with pp65 peptides enhanced in each case the capacity to induce the T-cell killing activity.

T cells primed by CD137L-DCs were at least twice as effective as T cells primed by classical DCs in lysing target cells at all E:T ratios tested. The percentages of specific lysis of target cells by T cells restimulated by untreated CD137L-DCs were 49 %, 63 % and 75 %, respectively, at E:T ratios of 5:1, 10:1 and 20:1, while the percentages were 16 %, 23 % and 35 %, respectively, for T cells primed by mature classical DCs. Taken together, these data demonstrate that autologous, antigen-specific T cells that have been restimulated by CD137L-DCs are functionally superior to T cells restimulated by classical DCs.

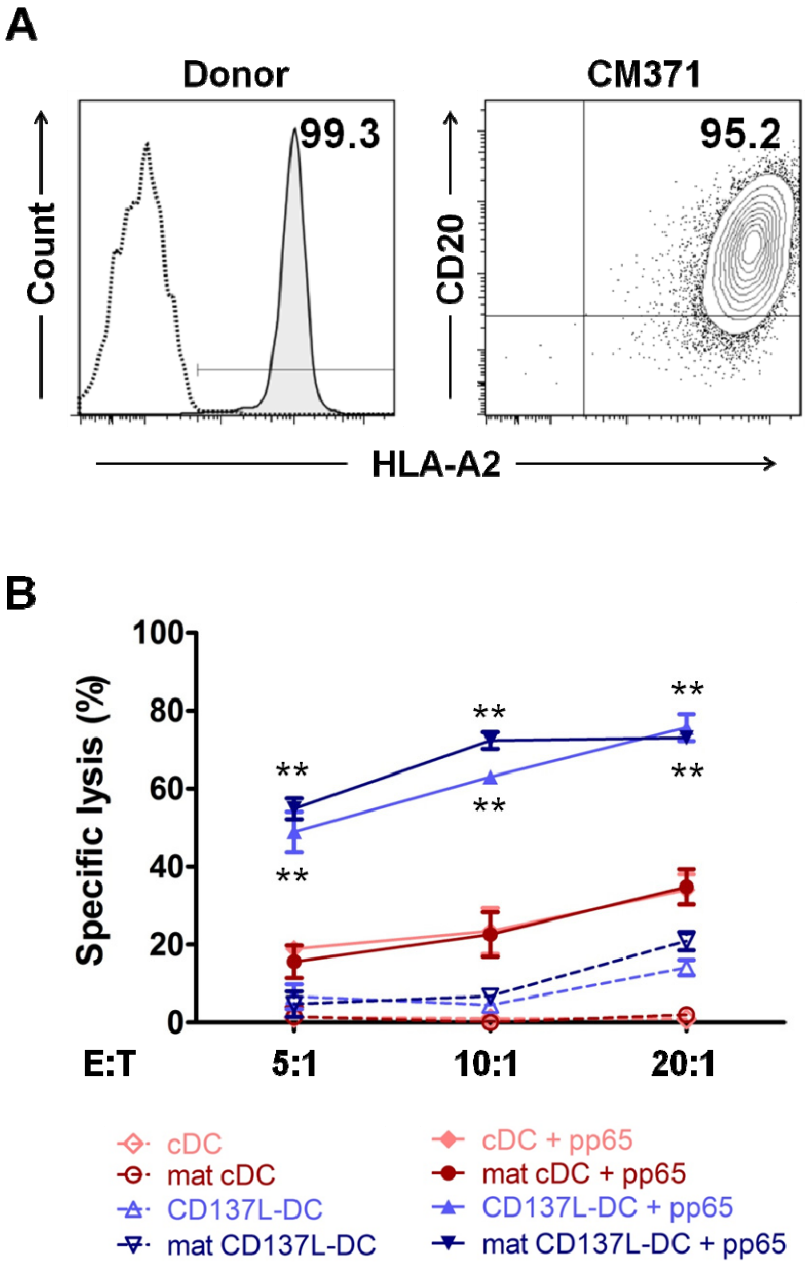


Figure 33. pp65 T cells restimulated by CD137L-DCs are more cytotoxic than T cells restimulated by classical DCs. (A) Monocytes were isolated from a HLA-A2^{+} donor, and the EBV-lymphoblastic cell line CM371 was immunostained for CD20 and HLA-A2 and analysed by flow cytometry. (B) CM371 cells were loaded with DELFIA® BATDA reagent, pulsed with the pp65 peptide pool and used as target cells. As a control, unpulsed cells were used. T cells restimulated by the different DCs for 5 days were added at the indicated effector/target ratios, and were incubated for 3 h. Depicted are mean ± standard deviation of percentages of specific lysis from triplicate measurements. $^{**}p<0.01$ using a two-tailed unpaired Student's *t*-test. These data are representative of at least two independent experiments with comparable results.

3.5. Transcriptional profiling of CD137L-DCs reveals a closer relationship with classical DCs rather than macrophages

To better understand the biological and functional properties of CD137L-DCs, we analysed their transcriptional profile and compared it to those of macrophages and classical DCs which are cellular subtypes representing terminal differentiation of monocytes.

Using monoytes from 4 healthy individuals, the various cellular subtypes; immature and mature classical DCs, macrophages, CD137L-DCs and Fc-treated monocytes, were generated over a period of 8 days. Gene expression profiles of these populations, together with that of a day 0 monocyte control, were obtained using Illumina Expression BeadChip arrays. Gene profiling of mature CD137L-DCs were not obtained as at that point of study, the optimised maturation factors (i.e. R848 plus IFN-γ) had not been deciphered. However, they were later used for protein expression verification studies and also functional assays.

To obtain differentially expressed genes, linear model for microarray data (limma) package with paired sample analysis was applied on the dataset (Smyth, 2004). Gene probes with *p*-values < 0.01 with respect to control monocytes were of interest. The CD137L-DC signature probes were selected by obtaining the gene set that was found in the intersection of differentially expressed genes of CD137-Fc treatment vs monocytes and differentially expressed genes of CD137-Fc vs Fc (figure 34). This allowed for the acquisition of differentially expressed genes which are contributed solely by CD137L reverse signalling into monocytes. A total of 974 probes, corresponding to 829 genes, were identified as CD137L-DC differentially expressed genes.

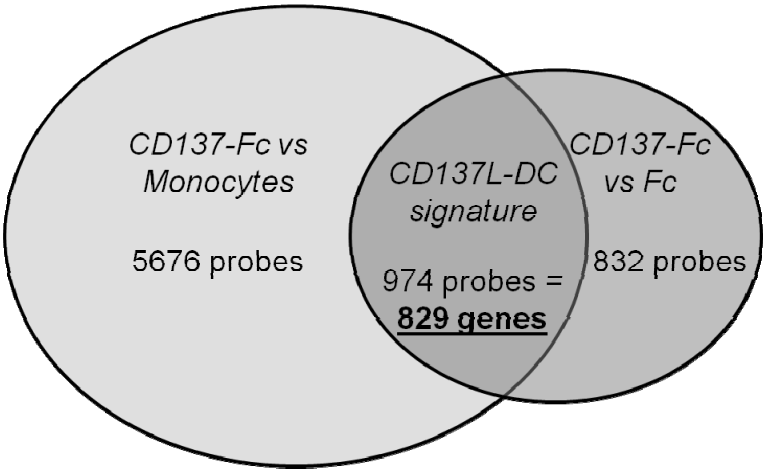
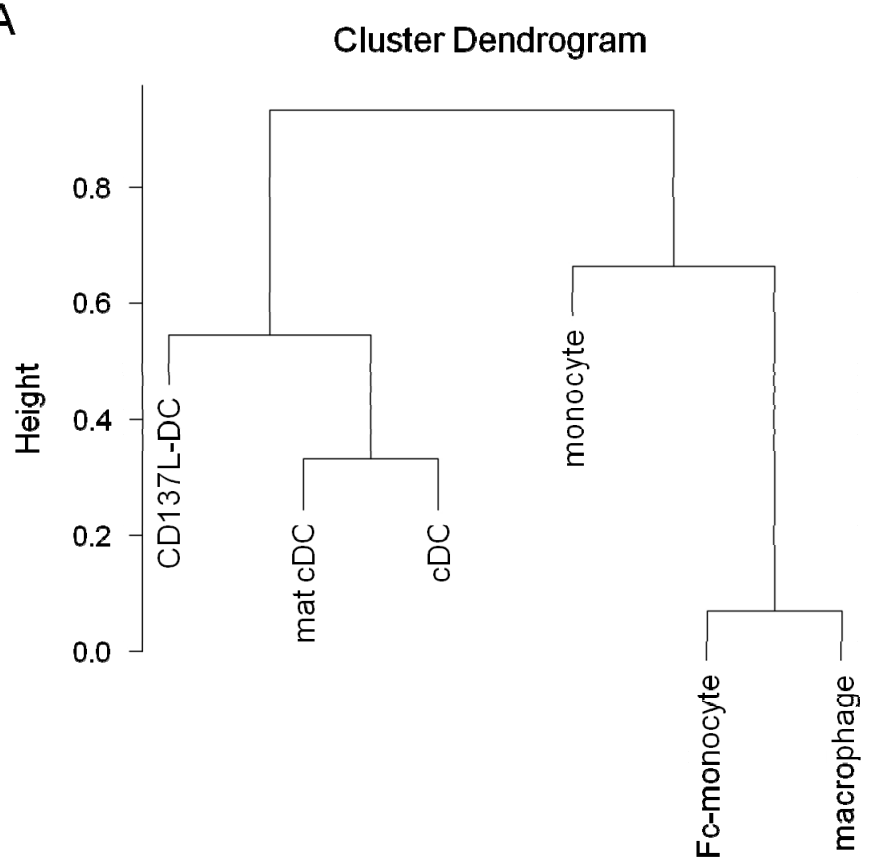


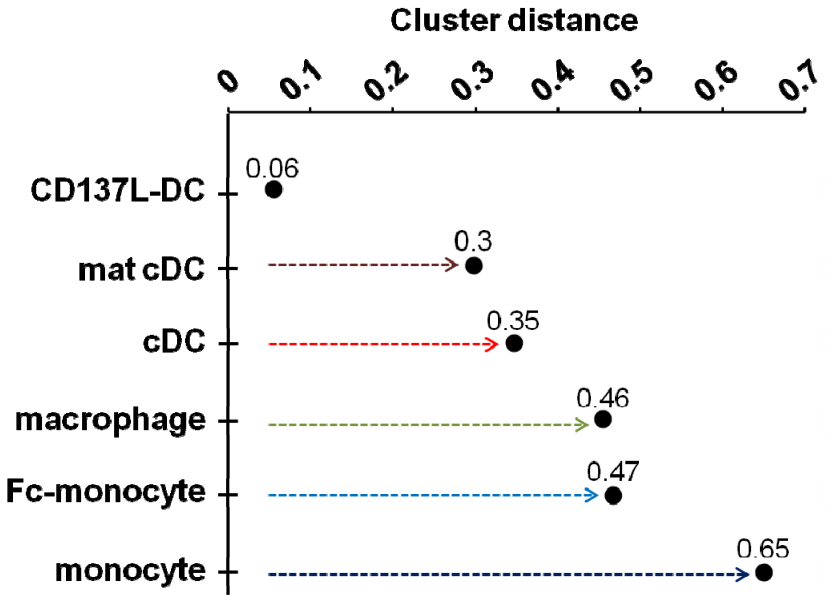
Figure 34. Methodology for the acquisition of CD137L-DC differentially expressed genes. The CD137L-DC signature probes were selected by obtaining the gene set that was found in the intersection of differentially expressed genes of CD137-Fc treatment vs monocytes and differentially expressed genes of CD137-Fc vs Fc. This allowed for the acquisition of differentially expressed genes which are contributed solely due to CD137L reverse signalling into monocytes.

To determine the relationship of CD137L-DCs with the other cell populations, the CD137L-DC differentially expressed genes dataset was subjected to hierarchical clustering and cMAP analysis (figure 35). The closer the cMAP score is to the value 1 the closer is the linkage to CD137L-DCs, while a larger cluster distance refers to a weaker linkage to CD137L-DCs. Both analyses unravelled that CD137L-DCs are more closely clustered to mature and immature classical DC than to macrophages. This reiterates the previous conclusion that CD137L reverse signalling drives monocytes towards DC differentiation.

A


Cluster Dendrogram
Height
0.0
0.2
0.4
0.6
0.8
CD137L-DC
mat cDC
cDC
monocyte
Fc-monocyte
macrophage

B


Cluster distance
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
CD137L-DC
mat cDC
cDC
macrophage
Fc-monocyte
monocyte
0.06
0.3
0.35
0.46
0.47
0.65

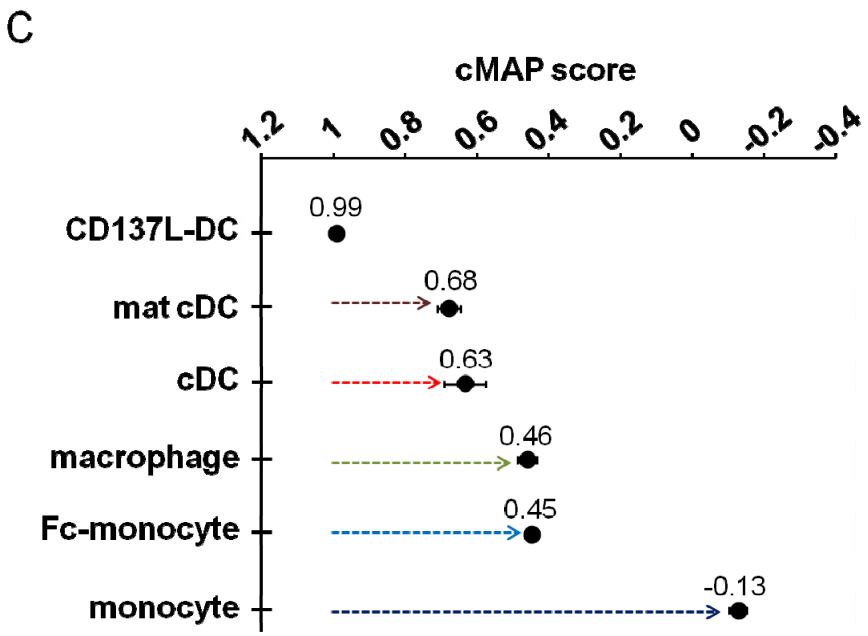


Figure 35. Transcriptional profiling shows that CD137L-DCs are more similar to classical DCs than macrophages. Indicated DCs and APCs were generated from 4 healthy donors. RNA was obtained and gene expression profiling was performed using Illumina Human HT12-V4 BeadChip arrays. Gene expression profiles of differentially expressed genes were subjected to (A) hierarchical clustering and (B) cluster distance analysis (Pearson correlation, average linkage between sample groups) using R package and also (C) cMAP analysis. The length of lines is proportional to the transcriptional similarity between CD137L-DCs and the respective DCs or APCs.

3.6. Characteristics of CD137L-DCs revealed by *in silico* analysis

The transcriptional fold-changes of the 829 signature CD137L-DC differentially expressed genes were compared alongside monocytes, macrophage, immature and mature classical DCs to acquire a list of genes which were highly expressed in CD137L-DCs with respect to the different cell population subtypes. The top 25 differentially expressed genes by CD137L-DCs in each group are shown in tables 7-10. To determine the unique functional characteristics of CD137L-DCs against each of the cell population, CD137L-DC differentially expressed genes with >2 fold change in each group

were subjected to Gene Ontology (GO) biologic process using DAVID tools (Huang da et al., 2009). Categories with significant enrichment (*p* value < 0.05) were acquired and similar categories were grouped together (table 11).

When compared to control monocytes, CD137L reverse signalling initiate functional responses that are distinctively involved in lipid processes, immune responses and adhesion (table 11). However, as CD137L-DCs are highly potent DCs, it was of interest to discover the key biologic functions that makes CD137L-DCs a stronger T-cell activator than classical DCs. To answer this, a GO comparison between CD137L-DCs with classical DCs was performed. GO enrichment of highly expressed differentially expressed genes in CD137L-DCs compared to mature classical DCs showed that CD137L-DCs participates greatly in cell adhesion, locomotion and activation of immune responses while functions of lipid processes, adhesion and immune responses are increased when compared to immature classical DCs. The representative genes involved in several of these GO enrichment categories are shown in figure 36. Hence, these biologic processes may be essential to unravel the reasons behind the heighten potency of CD137L-DCs compared to classical DCs.

Table 7: Top 25 differentially expressed genes in CD137L-DCs as compared to monocytes

GeneSymbol	Gene Name	Fold change
CCL22	Chemokine (C-C Motif) Ligand 22	2841
SPP1	Secreted Phosphoprotein 1	1575
MMP7	Matrix Metallopeptidase 7 (Matrilysin, Uterine)	841
GPNMB	Glycoprotein (Transmembrane) Nmb	547
CXCL5	Chemokine (C-X-C Motif) Ligand 5	545
LAMP3	Lysosomal-Associated Membrane Protein 3	458
TM4SF19	Transmembrane 4 L Six Family Member 19	419
MMP7	Matrix Metallopeptidase 7 (Matrilysin, Uterine)	392
INDO	Indoleamine 2,3-Dioxygenase 1	283
TM7SF4	Transmembrane 7 Superfamily Member 4	257
CYP27B1	Cytochrome P450, Family 27, Subfamily B, Polypeptide 1	196
SCG5	Secretogranin V (7B2 Protein)	192
LPL	Lipoprotein Lipase	191
FXYD2	Fxyd Domain Containing Ion Transport Regulator 2	185
CCL17	Chemokine (C-C Motif) Ligand 17	180
IDO1	Indoleamine 2,3-Dioxygenase 1	178
PLTP	Phospholipid Transfer Protein	159
SLCO2B1	Solute Carrier Organic Anion Transporter Family, Member 2B1	153
NDP	Norrie Disease (Pseudoglioma)	142
CCR7	Chemokine (C-C Motif) Receptor 7	124
TM4SF19	Transmembrane 4 L Six Family Member 19	119
PDPN	Podoplanin	118
DHCR24	24-Dehydrocholesterol Reductase	104
EBI3	Epstein-Barr Virus Induced 3	94

Table 8: Top 25 differentially expressed genes in CD137L-DCs as compared to mature classical DCs

GeneSymbol	GeneName	Fold change
CXCL5	Chemokine (C-X-C Motif) Ligand 5	392
CLEC5A	C-Type Lectin Domain Family 5, Member A	237
SCG5	Secretogranin V (7B2 Protein)	142
TNFRSF21	Tumor Necrosis Factor Receptor Superfamily, Member 21	142
C19orf59	Chromosome 19 Open Reading Frame 59	65
AOX1	Aldehyde Oxidase 1	53
GPC4	Glypican 4	43
MTMR11	Myotubularin Related Protein 11	35
NDRG2	Ndrg Family Member 2	33
SEZ6L2	Seizure Related 6 Homolog (Mouse)-Like 2	32
TPM2	Tropomyosin 2 (Beta)	30
FOS	V-Fos Fbj Murine Osteosarcoma Viral Oncogene Homolog	28
DBN1	Drebrin 1	26
PTPRF	Protein Tyrosine Phosphatase, Receptor Type, F	26
CA2	Carbonic Anhydrase Ii	25
MYO1B	Myosin Ib	25
PITPNC1	Phosphatidylinositol Transfer Protein, Cytoplasmic 1	24

HOPX	Hop Homeobox	24
NRGN	Neurogranin (Protein Kinase C Substrate, Rc3)	24
TACSTD2	Tumor-Associated Calcium Signal Transducer 2	22
GREM1	Gremlin 1, Cysteine Knot Superfamily, Homolog (Xenopus Laevis)	21
PTPN13	Protein Tyrosine Phosphatase, Non-Receptor Type 13 (Apo-1/Cd95 (Fas)-Associated Phosphatase)	20
IGFBP6	Insulin-Like Growth Factor Binding Protein 6	19
FYN	Fyn Oncogene Related To Src, Fgr, Yes	18
COL22A1	Collagen, Type Xxii, Alpha 1	18

Table 9: Top 25 differentially expressed genes in CD137L-DCs as compared to immature classical DCs

GeneSymbol	GeneName	Fold change
CXCL5	Chemokine (C-X-C Motif) Ligand 5	842
INDO	Indoleamine 2,3-Dioxygenase 1	461
MMP7	Matrix Metallopeptidase 7 (Matrilysin, Uterine)	294
CLEC5A	C-Type Lectin Domain Family 5, Member A	175
SCG5	Secretogranin V (7B2 Protein)	155
CCR7	Chemokine (C-C Motif) Receptor 7	114
CLLU1OS	Chronic Lymphocytic Leukemia Up-Regulated 1 Opposite Strand	65
EBI3	Epstein-Barr Virus Induced 3	61
GPC4	Glypican 4	55
AOX1	Aldehyde Oxidase 1	53
TMEM132A	Transmembrane Protein 132A	41
PTPRF	Protein Tyrosine Phosphatase, Receptor Type, F	38
GLDN	Gliomedin	36
PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1	36
SLC1A2	Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter), Member 2	33
SEZ6L2	Seizure Related 6 Homolog (Mouse)-Like 2	28
FPR2	Formyl Peptide Receptor 2	28
MYO1B	Myosin Ib	26
DBN1	Drebrin 1	25
LAT	Linker For Activation Of T cells	23
PDPN	Podoplanin	23
FSTL1	Follistatin-Like 1	22
TM4SF1	Transmembrane 4 L Six Family Member 1	22
MTMR11	Myotubularin Related Protein 11	21
BIRC3	Baculoviral Iap Repeat-Containing 3	21

Table 10: Top 25 differentially expressed genes in CD137L-DCs as compared to macrophages

GeneSymbol	GeneName	Fold change
CCL22	Chemokine (C-C Motif) Ligand 22	2727
INDO	Indoleamine 2,3-Dioxygenase 1	413

LAMP3	Lysosomal-Associated Membrane Protein 3	333
TACSTD2	Tumor-Associated Calcium Signal Transducer 2	277
MMP7	Matrix Metallopeptidase 7 (Matrilysin, Uterine)	275
GPC4	Glypican 4	213
CYP27B1	Cytochrome P450, Family 27, Subfamily B, Polypeptide 1	203
LPL	Lipoprotein Lipase	177
CCL17	Chemokine (C-C Motif) Ligand 17	176
CCR7	Chemokine (C-C Motif) Receptor 7	144
NDP	Norrie Disease (Pseudoglioma)	139
NDRG2	Ndrg Family Member 2	99
SPOCD1	Spoc Domain Containing 1	93
EBI3	Epstein-Barr Virus Induced 3	73
CCND1	Cyclin D1	64
ALDH1A2	Aldehyde Dehydrogenase 1 Family, Member A2	45
SLC1A2	Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter), Member 2	44
CLLU1OS	Chronic Lymphocytic Leukemia Up-Regulated 1 Opposite Strand	42
SERPINE1	Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1), Member 1	38
CXCL5	Chemokine (C-X-C Motif) Ligand 5	36
PHLDA3	Pleckstrin Homology-Like Domain, Family A, Member 3	31
PSD3	Pleckstrin And Sec7 Domain Containing 3	30
MATK	Megakaryocyte-Associated Tyrosine Kinase	29
TMEM132A	Transmembrane Protein 132A	28
ZNF366	Zinc Finger Protein 366	27

Table 11: GO enrichment of biologic processes of CD137L-DCs compared to other DCs/APCs.

Term	**Count**	***p***
CD137L-DC differentially expressed genes which are ≥ 2× fold-change compared to monocytes		
Lipid processes		
GO:0016126: sterol biosynthetic process	14	2.4E-12
GO:0006694: steroid biosynthetic process	19	7.0E-12
GO:0006695: cholesterol biosynthetic process	11	5.7E-10
GO:0008610: lipid biosynthetic process	32	6.2E-10
GO:0008202: steroid metabolic process	24	4.3E-9
GO:0016125: sterol metabolic process	17	9.3E-9
GO:0008203: cholesterol metabolic process	16	1.8E-8
Immune response		
GO:0009611: response to wounding	34	5.1E-6
GO:0006954: inflammatory response	19	2.8E-3
GO:0006952: defense response	26	2.3E-2
Adhesion		
GO:0045785: positive regulation of cell adhesion	6	2.1E-2
GO:0022407: regulation of cell adhesion	9	3.0E-2
GO:0010810: regulation of cell-substrate adhesion	5	3.3E-2
GO:0010811: positive regulation of cell-substrate adhesion	4	3.7E-2
GO:0022610: biological adhesion	29	2.1E-2
GO:0007155: cell adhesion	29	2.1E-2

CD137L-DC differentially expressed genes which are ≥ 2× fold-change compared to mature classical DC		
Adhesion		
GO:0007155: cell adhesion	24	2.0E-5
GO:0022610: biological adhesion	24	2.1E-5
Locomotion		
GO:0042330: taxis	9	9.0E-4
GO:0006935: chemotaxis	9	9.0E-4
GO:0007610: behavior	15	2.3E-3
GO:0007626: locomotory behavior	10	7.5E-3
Immune response		
GO:0009611: response to wounding	20	3.5E-5
GO:0006954: inflammatory response	10	2.1E-2
GO:0006952: defense response	13	8.4E-2
CD137L-DC differentially expressed genes which are ≥ 2× fold-change compared to immature classical DC		
Lipid processes		
GO:0010743: regulation of foam cell differentiation	5	2.7E-4
GO:0010885: regulation of cholesterol storage	4	3.2E-4
GO:0010883: regulation of lipid storage	4	2.3E-3
Adhesion		
GO:0007155: cell adhesion	21	6.8E-4
GO:0022610: biological adhesion	21	6.9E-4
Immune response		
GO:0009611: response to wounding	22	4.5E-6
GO:0006954: inflammatory response	12	3.2E-3
GO:0006952: defense response	15	2.7E-2
CD137L-DC differentially expressed genes which are ≥ 2× fold-change compared to macrophage		
Lipid processes		
GO:0016126: sterol biosynthetic process	10	1.5E-10
GO:0006694: steroid biosynthetic process	12	3.2E-9
GO:0006695: cholesterol biosynthetic process	8	1.2E-8
GO:0016125: sterol metabolic process	10	2.3E-6
GO:0008202: steroid metabolic process	13	3.6E-6
GO:0008610: lipid biosynthetic process	16	4.5E-6
GO:0008203: cholesterol metabolic process	9	1.0E-5
GO:0008299: isoprenoid biosynthetic process	5	7.0E-5
GO:0055114: oxidation reduction	20	1.3E-4
GO:0006720: isoprenoid metabolic process	5	1.6E-3
GO:0010743: regulation of foam cell differentiation	4	2.8E-3
GO:0010885: regulation of cholesterol storage	3	6.7E-3
GO:0010883: regulation of lipid storage	3	2.4E-2
Immune response		
GO:0009611: response to wounding	16	1.1E-3
GO:0006952: defense response	14	2.4E-2
GO:0006954: inflammatory response	9	3.4E-2
GO:0070663: regulation of leukocyte proliferation	5	1.6E-2
GO:0032944: regulation of mononuclear cell proliferation	5	1.6E-2
GO:0051249: regulation of lymphocyte activation	6	2.8E-2
GO:0019955: cytokine binding	5	3.7E-2
GO:0002694: regulation of leukocyte activation	6	4.2E-2
Growth		
GO:0040007: growth	7	1.9E-2
GO:0031099: regeneration	4	4.5E-2

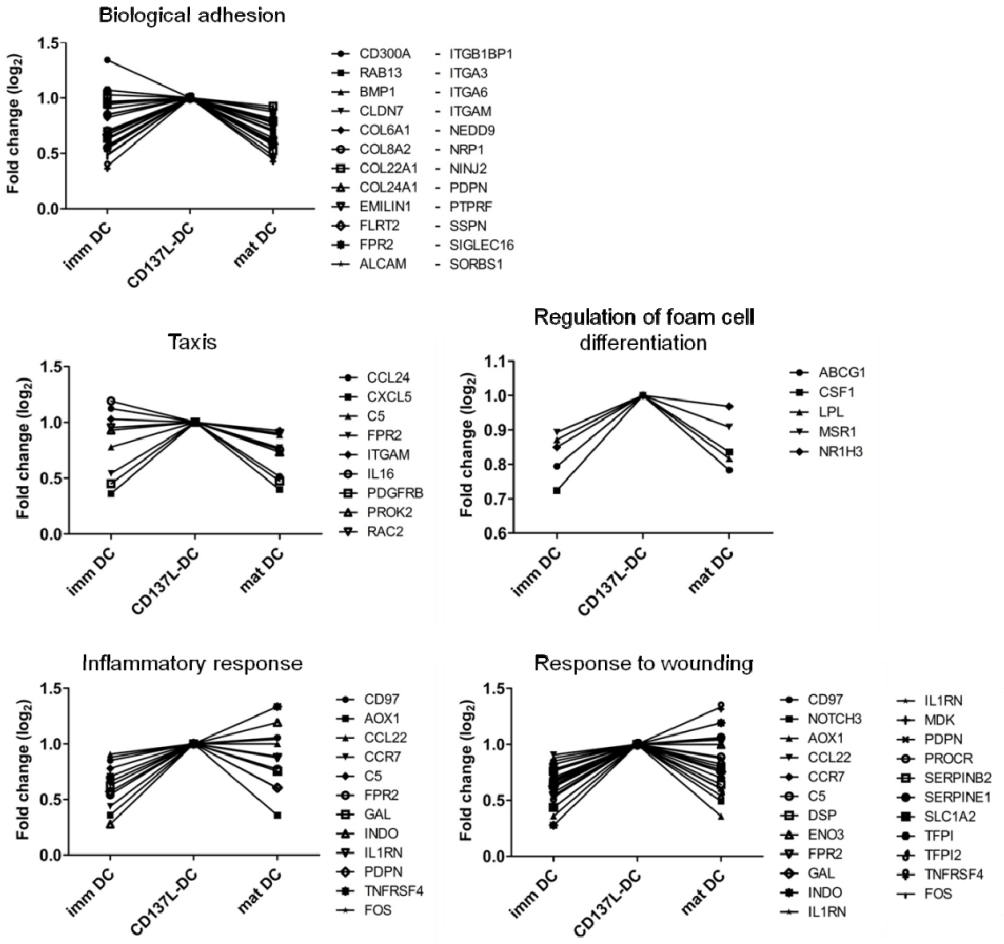


Figure 36. Representative GO categories associated with the differentially expressed genes most highly expressed by CD137L-DCs. The log 2 levels of genes associated with various GO categories deemed to be functionally upregulated by CD137L-DCs using DAVID tools. Fold change normalized to the CD137L-DC subset are shown.

3.6.1 Flow cytometric analysis of surface markers expressed by CD137L-DCs

GO enrichment analysis revealed that CD137L-DCs have several enhanced biological functions that may in turn positively influence their interaction and activation of T cells. Of primary interest were their adhesion properties.

In order for T cells to mount a successful response, it is essential that these cells interact with APCs, form stable immune synapses and are subjected to appropriate MHC-peptide complexes and co-stimulatory molecules (Thauland and Parker, 2010). Structural durability of the immune synapse is key and it has been shown that stable and longer-lasting synapses correlate with the development of stronger immune responses (Hugues et al., 2004). Correlating effective formation of the immune synapse with the superior adhesion properties of CD137L-DCs, it is hypothesised that perhaps this could lead to a more stable immune synapse and therefore their superior T-cell activation ability.

Intracellular adhesion molecule 1 (ICAM-1) forms a ring on the outer part of the immune synapse and is necessary for the initial contact with T cells via interaction with LFA-1 (Davis, 2009). Although not considered as a CD137L-DC signature gene, the surface expression of ICAM-1 was determined. Virtually all CD137L-DCs expressed ICAM-1 at similar levels with immature classical DCs and macrophages while mature CD137L-DCs expressed 3.5 times and mature classical DCs expressed 7 times more ICAM-1 (figure 37B). This corresponded to the mRNA transcriptional profile (figure 37A). Another cell adhesion molecule, ALCAM, not only interacts with T cells to maintain a stable contact but also acts as a ligand to the co-stimulatory molecule CD6 on T cells (Zimmerman et al., 2006). ALCAM is a CD137L-DC signature gene

(figures 36 and 37A) and correspondingly, it is highly expressed on CD137L-DCs (99 %, MFI= 962) while maturation with R848 plus IFN-γ only slightly increased its expression (98 %, MFI= 1151), (figure 37B). Expression is also high in classical DCs but only upon their maturation (97 %, MFI= 943). Other adhesion molecules such as PDPN and CLEC5A are expressed on CD137L-DCs but are significantly less on both mature and immature classical DCs (PDPN; 33 % vs 16 % vs 1 %, respectively, and CLEC5A; 72 % vs 18 % vs 2 %, respectively).

In addition, surface markers which are involved in inflammatory responses, and in particular co-stimulation, were also investigated. OX40L and CD70 are members of the TNF superfamily and are important co-stimulatory molecules commonly found on mature classical DCs (Kober et al., 2008; Krause et al., 2009). Based on the transcriptional profile, untreated CD137L-DCs express these genes at a higher levels than mature classical DCs, however, this was not the case when their surface expression was determined (figure 37). Untreated CD137L-DCs did not express both OX40L and CD70 while the latter was expressed only upon their maturation (42 %, MFI= 273). Similarly, 39 % of mature classical DCs expressed CD70 (MFI= 300) but not OX40L. CD83, a mature DC marker, was detected on 38 % of untreated CD137L-DCs while only 12 % of immature DCs expressed this protein.

The co-stimulatory molecule profiles are similar between immature classical DCs and untreated CD137L-DCs (with the exception of heightened CD83 in the latter) and also between mature classical DCs and mature CD137L-DCs. Taken together, it is plausible to propose that CD137L-DCs exert their superior T-cell activation ability due to their heighten adhesion properties rather than their expression of co-stimulatory molecules.

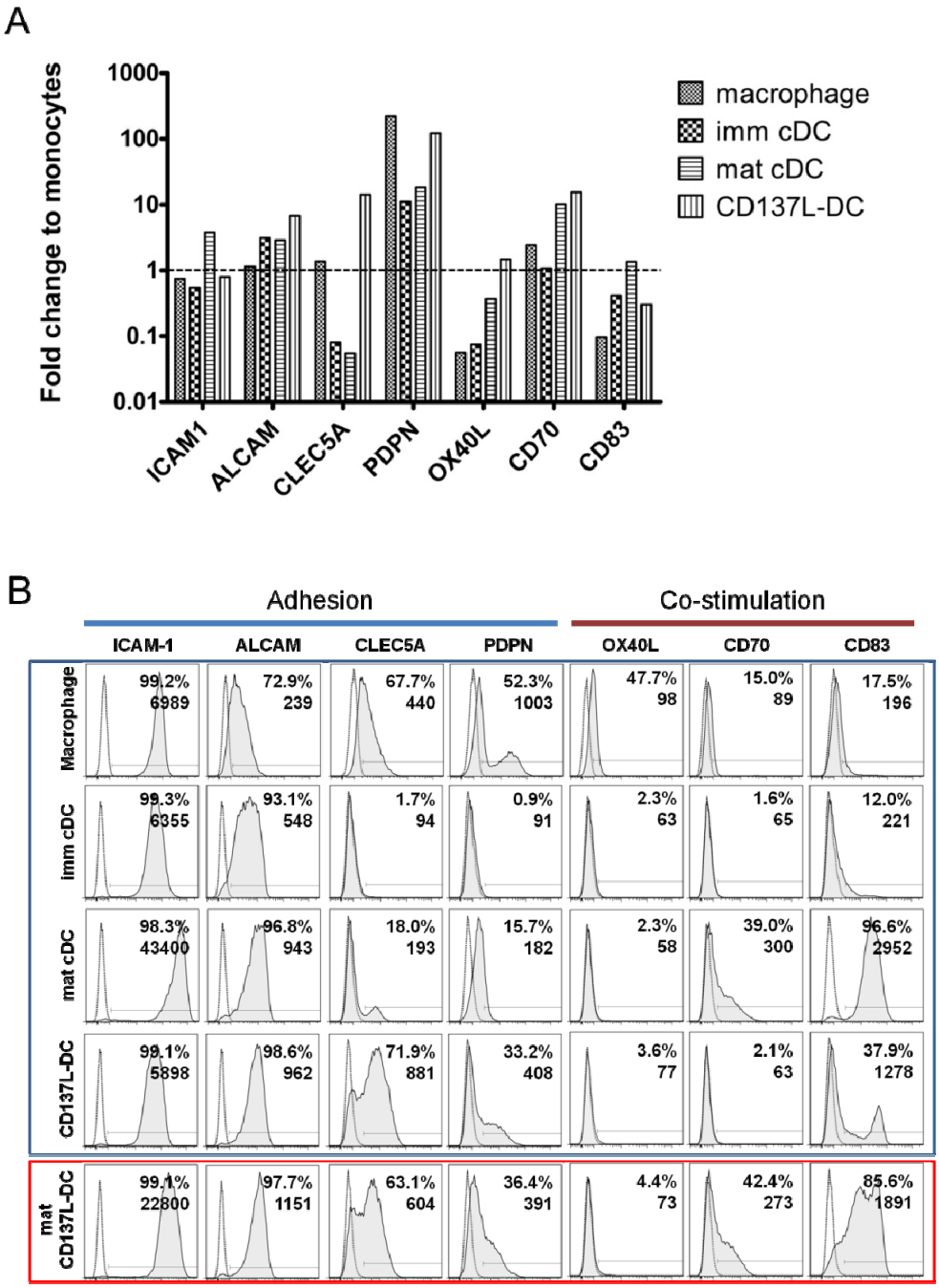


Figure 37. Transcriptional expression and corresponding flow cytometric analysis of selected adhesion and co-stimulatory molecules. (A) Fold change of transcriptional expression of various adhesion and co-stimulatory genes as compared to baseline monocyte expression levels. The horizontal dotted line refers to transcriptional levels of monocytes. Mature CD137L-DCs were not available for transcriptional analysis. (B) DCs and APCs were generated for 6 days and were further treated with LPS plus IFN-γ to generate mature classical DCs or R848 plus IFN-γ to generate mature CD137L-DCs for 18 h. Cells were harvested, immunostained for the described corresponding markers, and analysed by flow cytometry. Values in histograms depict percentages and MFI of a single experiment. These data are representative of at least two independent experiments with comparable results.

3.7. The biological contributions of various CD137L-DC differentially expressed genes

CD137L-DCs express certain proteins such as ALCAM and other adhesion molecules that may contribute towards their general biological function and also T-cell activating potential. To test this hypothesis, we performed several functional assays namely to quantify adherence ability of CD137L-DCs and the requirement of ALCAM for T-cell activation.

3.7.1. Attachment/detachment assay

As many as 24 genes involved in adhesion were more highly transcriptionally expressed on untreated CD137L-DCs than their classical counterparts (figure 36). To verify if this upregulation indeed translates to a stronger adhesion capacity, CD137L-DCs were tested for their ability to re-adhere onto cell culture plates within a short period. Day 7 DCs were harvested and re-seeded onto culture plates before suspended cells and adhered cells were counted using flow cytometric analysis at various time-points.

As expected, CD137L-DCs were able to adhere effectively even within a short time span of 10 min upon inoculation. Maximal adherence had already been achieved within this period as there was virtually no time-dependent increase in the percentage of CD137L-DCs adhered (10 min: 94 %, 45 min: 95 %, 90 min: 97 %), (figure 38A). Mature CD137L-DCs were however, slightly less adherent than untreated CD137L-DCs within the stipulated time-points tested. Immature classical DCs were the least adherent cells as only 53 % adhered within 10 min while mature classical DCs had intermediate adherent properties with 65 % of cells being adherent. Interestingly, both immature and mature classical DCs were less attached at 45 min (38 % and 58 %,

respectively) but regained adherence at 90 min (43 % and 66 %, respectively).

Having shown that CD137L-DCs do adhere to culture plates at a faster pace than the classical DCs, we next tested the strength of adherence. Equal numbers of various types of DCs were plated onto culture wells before being incubated overnight to allow for complete attachment. DCs were then detached by trypsin-EDTA treatment for various time periods and counted. Concurring with the abovementioned observations, CD137L-DCs, both untreated and matured, do indeed have strong adherent properties. Only about 5 % of the total CD137L-DCs were detached from the culture plate at 10 min, and prolonged periods of trypsin-EDTA treatment failed to further detach the DCs (figure 38B). Despite having an initial intermediate adhesion characteristic, mature classical DCs were most easily detached by trypsin-EDTA (32-34 % detachment). On the other hand, 19-22 % of immature classical DCs were detached upon treatment. Taken together, the functional observations are consistent with the GO enrichment data regarding heighten adhesion properties of CD137L-DCs.

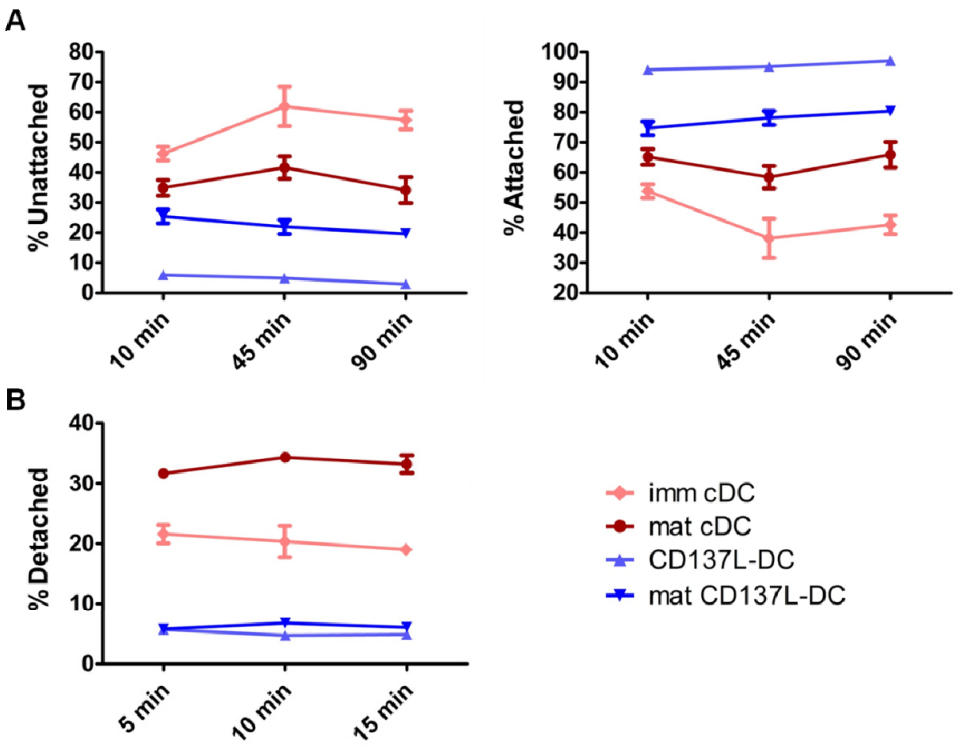


Figure 38. CD137L-DCs are highly adherent cells. DCs were generated for a period of 7 days with classical DCs matured by LPS plus IFN-γ while CD137L-DCs by R848 plus IFN-γ for the final 18 h. DCs were harvested and seeded at equal density on culture plate wells. (A) DCs were incubated at 37ºC and at various time-points, suspended (unattached) and adhered (attached) cells were harvested and counted using counting beads. Depicted are means ± standard deviations of percentages of unattached and attached DC from duplicate samples. (B) DCs were incubated at 37ºC overnight before cells were treated with trypsin-EDTA for 5, 10, and 15 min. Detached DCs were harvested and counted using counting beads. Depicted are mean percentages ± standard deviations of detached DCs from duplicate samples. These data are representative of two independent experiments with comparable results.

3.7.2. ALCAM neutralising assay

Amongst the genes involved in adhesion, ALCAM was a prime candidate for further studies as it has been described to not only play a role in DC-T-cell adhesion but also in lasting T-cell activation via its interaction with CD6 on T cells (Zimmerman et al., 2006). Since CD137L-DCs were able to strongly activate antigen-specific T cells even after 5 days of co-culture (figures 30 and 31), it was speculated that ALCAM may be an important factor that contributes to long-term T-cell activation.

To elucidate the contribution of DC-expressing ALCAM on T-cell activities, neutralising studies were performed. Noteworthy, ALCAM has been reported to be expressed also on activated lymphocytes (Bowen et al., 2000) and therefore we confirmed this by flow cytometry prior to initiating blocking studies. In the absence of mitogens, resting T cells are devoid of ALCAM expression even after 48 h of culture (figure 39). Nonetheless, activation of T cells by PMA plus ionomycin was able induce low expression of ALCAM on 11 % of T cells although the MFI remained very low at 32. As a whole, activated T cells do not express high amounts of ALCAM, thus subsequent blocking of ALCAM with antagonistic anti-ALCAM antibodies in co-culture studies should predominantly affect ALCAM on CD137L-DCs.

Neutralisation of ALCAM on untreated CD137L-DCs led to modest reduction in T-cell proliferation (12 % reduction), (figure 40). This effect was more pronounced when ALCAM on mature CD137L-DCs were neutralised. In this case, T-cell proliferation was reduced by nearly 40 %. Interestingly, despite the presence of ALCAM on immature and mature classical DCs, there were no effects on T-cell proliferation even after the blockade of ALCAM with

antagonistic antibody. These evidences suggest that ALCAM may play a role in the ability of CD137L-DCs to promote potent T-cell activation and function.

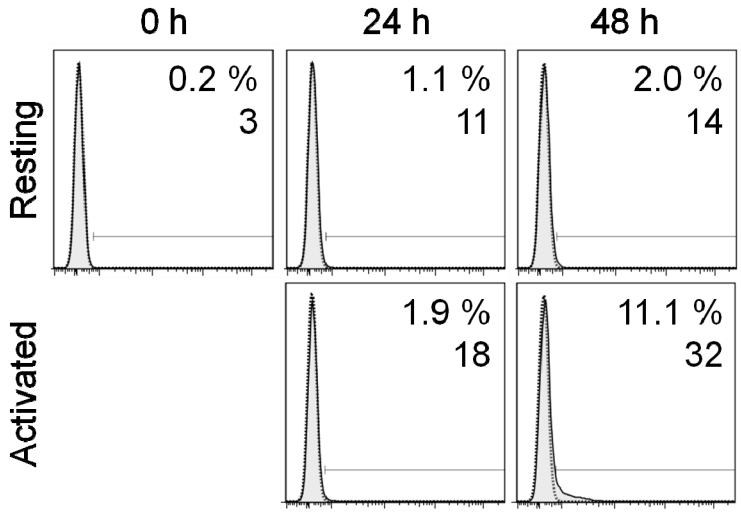


Figure 39. Activated T cells express low levels of ALCAM. T cells were isolated from PBMCs by positive selection and treated with PMA plus ionomycin for 24 and 48 h to induce activation. Resting T cells were used as controls. Cells were immunostained for surface ALCAM and analysed by flow cytometry. Unshaded and grey histograms represent unstained control and ALCAM, respectively. Values in histograms are percentages of positive cells and MFI. This experiment has been performed twice with comparable results.

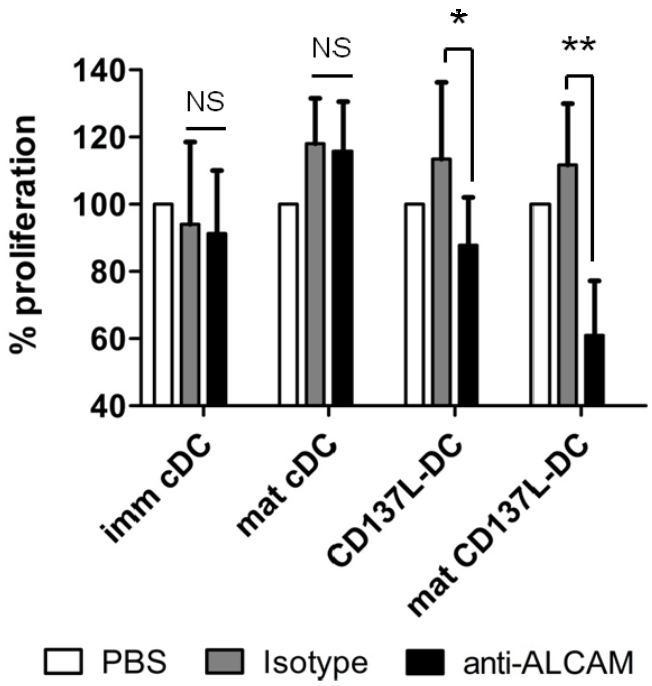


Figure 40. Neutralising ALCAM on CD137L-DCs reduces T-cell activation. DCs were generated for a period of 7 days with classical DCs matured by LPS plus IFN-γ while CD137L-DCs by R848 plus IFN-γ for the final 18 h. DCs were then further co-cultured with allogeneic T cells at a ratio of 1:10. At day 4, proliferation was quantified by ^{3}H-thymidine incorporation. Proliferations are expressed as the percentage of PBS control and are means ± standard deviations of triplicate measurements. *p<0.05; **p<0.01 using a two-tailed unpaired Student's t-test. This experiment has been performed twice with comparable results.

CHAPTER 4: DISCUSSION

4.1. Discovery of a new form of DC for immunotherapy: CD137L-DCs

The traditional oncologic treatment modalities – surgery, chemotherapy and radiotherapy – are the status quo regimes for the combat against cancer. More recently, biologic therapies utilising the immune system are gaining acceptance as a complementary approach towards a more holistic form of anti-cancer treatment. Specifically, immunotherapeutic procedures such as infusing immune modulating antibodies, NK and T-cell adoptive transfer, and DC-based vaccines are some avenues in which scientists are probing to develop (Childs and Berg, 2013; Kalos and June, 2013; Palucka and Banchereau, 2013; Sliwkowski and Mellman, 2013). Though much insights and progress have been gained, it is still early days in the immunotherapeutic field and much research is required to understand and optimise the protocols for treatment.

In this project, we aimed to further develop DC-based vaccines by characterising a novel type of DC that was previously described to be biologically potent in instilling allogeneic T-cell responses (Ju et al., 2009; Kwajah and Schwarz, 2010). Nonetheless, these CD137L-stimulated DCs (CD137L-DCs) had not been tested for their ability to stimulate autologous T cells. Therefore, it is paramount that these CD137L-DCs be further defined in an autologous system in order to determine their potential use as an immunological tool for therapy. Through the use to cytomegalovirus (CMV)-derived protein pp65 as a model antigen, we demonstrated that CD137L-DCs induce an abundant secretion of IFN-γ, TNF and IL-13 from autologous pp65-specific T cells, endowing them with a robust cytotoxic potential towards HLA-matched, pp65-pulsed target cells. Furthermore, transcriptional profiling of

CD137L-DCs corroborated with their biological phenotype in which they are more similar to classical DCs than to macrophages. In this section, we will discuss the functional biology of CD137L-DCs in addition to their requirements for maturation and activation. Next, we will evaluate the potential of CD137L-DCs as a means for DC-based immunotherapy and consider further clarifications required before any application in the clinical setting can be envisaged.

4.2. *Ex vivo* generated DC-based vaccines: A challenge

Appreciable effort has been made to develop strategies for using *ex-vivo* generated DCs to mount a proficient anti-tumour immune response. Many clinical studies from the past decade have concluded that such vaccines are safe and generally have a low toxicity profile thus making the procedure an enticing option for cancer treatment (Alatrash et al., 2013). Conventional GM-CSF plus IL-4 generated DCs (classical DCs) are intensively researched upon and have been successful in the clinic although the response rates of patients to DC-based therapies remain low (Figdor et al., 2004; Osada et al., 2006; Thomas-Kaskel and Veelken, 2007; Tuyaerts et al., 2007). More recently, further developments have implemented new innovations in DC preparations such as by inducing the differentiation of DCs within three days and those matured by TLR agonists to facilitate production of IL-12 (Frankenberger and Schendel, 2012). Although many current protocols are able to stimulate tumour-specific $CD4^+$ and/or $CD8^+$ T cells, most responses are neither robust nor long lasting owing to inadequate T-cell activation and maintenance coupled with a highly immunosuppressive tumour microenvironment (Hadrup et al., 2013). Further, a major regulatory obstacle for immunotherapy is the requirement of good manufacturing production

(GMP). In addition to the need to use GMP-grade media and antigens (peptide/proteins or RNA), the majority of current DC production and maturation protocols require the application of a large amount of recombinant cytokines including GM-CSF, IL-4, IL-1β, IL-6, IFN-α, TNF and PGE2 which in turn adds to the complexity of the protocol and an increase in production costs (Cintolo et al., 2012; Erdmann and Schuler-Thurner, 2010). Thus, there is presently an immense interest in the aspects of modulating DCs in terms of their development, maturation, antigen delivery and mode of vaccine inoculation (Cintolo et al., 2012; Palucka and Banchereau, 2013; Schuler, 2010). Against a backdrop of intensive exploration for optimised DC-based vaccine protocols, our identification and characterisation of this novel yet potent CD137L-DCs is particularly pertinent.

4.3. Activation of CD137L-DCs by exogenous cytokines

As reviewed in the introductory section, T cells can be polarised towards various phenotypes (i.e. Th1, Th2, Th17, Treg, etc) and this is dependent on the activation status of the stimulating DCs. Such differentially activated DCs are developed in the presence of inflammatory cytokines and mediating factors.

Before proceeding to the maturation experiments, preliminary findings suggested that CD137L-DCs are more potent than classical DCs when peptides are used. Although IFN-γ production by T cells restimulated by CD137L-DCs was higher than those restimulated by classical DCs, there was also no difference in their ability to promote stronger antigen-specific T-cell proliferation (figures 8-10). It is, however, important to note that the detection of IFN-γ production by T cells and their proliferation were performed at a

different time-points (18 h for IFN-γ production and 5 days for proliferation). Such a discrepancy could be due to the difference in kinetics between the onset of cytokine production and proliferation. Additionally, CD137L-DCs were weaker T-cell activators compared to classical DCs when whole protein antigens, in this case tetanus toxoid, were used as an antigen of interest (figures 11 and 12). Even the addition of LPS or TNF which are commonly used to induce type-1 DC maturation failed to further activate CD137L-DCs (figures 11 and 12), (Kalinski et al., 2009). In fact, pre-treatment of CD137L-DCs with either LPS or TNF rendered the peptide-restimulated T cells less able to secrete IFN-γ (figure 9). These initial findings prompted us to search for maturation factors that may be able to potentiate the T-cell activating effects of CD137L-DCs.

It was previously described that CD137L-DCs are able to induce a stronger T-cell response than mature classical DCs (Ju et al., 2009; Kwajah and Schwarz, 2010). In order to further boost T-cell activation, Kwajah and Schwarz further treated CD137L-DCs with a combination of LPS (a TLR4 agonist) and IFN-γ, a commonly used DC maturation cocktail. Although this concoction led to a substantial increase in the expression of CD80, CD86, HLA-DR and CCR7 on CD137L-DCs, therefore prompting a matured identity, the resultant mature CD137L-DCs were unable to potentiate allogeneic T-cell activation, and were essentially similar to untreated CD137L-DCs (Kwajah and Schwarz, 2010). Nevertheless, the fact that the expression of DC maturation markers could be upregulated implied that CD137L-DCs can be enhanced by maturation, but it was unclear whether factors other than, or in addition to, LPS and IFN-γ would be required. Accordingly, we explored the possibility to augment the T-cell activating potency of CD137L-DCs by using an array of published maturation cocktails (table 4), (Boullart et al., 2008;

Jonuleit et al., 1997; Zobywalski et al., 2007). These cytokine cocktails were carefully selected for their ability to promote potent Th1 and $CD8^+$ CTL responses which are desirable for anti-tumour therapy.

Amongst the 7 different types of maturation cocktails used, cocktails C3 and C4 were able to promote further morphological differences as compared to the untreated CD137L-DCs (figure 13). In either C3 or C4 matured conditions, CD137L-DCs were much more adhered and had more pronounced dendrite and spindle formation. Such morphological changes were the first evidence to propose that these maturation cocktails were able to further activate CD137L-DCs. Also, the concomitant increase in cellular adhesion was suggestive of a more activated phenotype as the adherent CD137L-DCs, rather than those in suspension, were defined to be the population that contributes significantly to the T-cell activation (personal communication with Dr. Shaqireen Kwajah).

Furthermore, CD137L-DCs in the presence of maturation cocktails C3 and C4 had significantly higher expression of DC maturation marker CD83, co-stimulatory molecule CD86, and HLA-DR while CD80 and CCR7, an essential chemokine receptor required to guide DC migration to the lymph nodes, were slightly upregulated (figure 15), (Gunn, 2003; Randolph et al., 2005). These results supplemented the morphological changes observed in C3 and C4-treated CD137L-DCs. A salient observation was that the abovementioned surface molecules were also upregulated in C1 (LPS and IFN-γ)-treated CD137L-DCs (figure 15) and as previously discussed, these maturation factors were not able to potentiate T-cell stimulation despite the apparent maturation state of CD137L-DCs (Kwajah and Schwarz, 2010). Indeed, our data supports this view as C1-treated CD137L-DCs have a similar capacity as their untreated counterparts to induce T-cell activation (figure 17). Thus, a supposed mature DC phenotype based on the expression of surface

molecules may not necessarily translate to a more biologically potent activity. It is therefore essential that the DCs' functional potential be ascertained by their ability to activate T cells rather than solely relying on maturation markers.

That said, the effects of maturing CD137L-DCs by C3 or C4 was determined to be biologically functional as either cocktail was able to potentiate their T-cell stimulation ability as evidenced by an increase in T-cell proliferation and expression of IFN-γ (figure 17). Such a gain in the DC potency is highly desirable as DC-based vaccines rely tremendously on the ability to promote strong antigen-specific T-cell expansion in the tumour-bearing individual (Kalinski et al., 2009). Quantity apart, the generated vaccine-elicited T cells must support anti-tumour activities and a major criterion is for the T cells to be able to secrete high amounts of IFN-γ. The production of this cytokine can bolster a Th1 response and concurrently promote effector $CD8^+$ CTL function (Smeltz et al., 2002). As expected, culturing allogeneic T cells with either C3 or C4-treated CD137L-DCs drove substantial production of IFN-γ by more than 2-fold compared to untreated CD137L-DCs, and this supports the notion of a more mature and active anti-tumour DC phenotype (figure 17).

More often than not, a potent Th1 response and the associated IFN-γ secretion by T cells are due to the release of soluble factors such as cytokines by DCs. This contributes to signal 3 which instigates the polarisation of T cells towards various subsets including Th1, Th2, and Th17 (de Jong et al., 2005; Kalinski et al., 1999). Among the most well studied DC-derived pro-inflammatory cytokine is IL-12 whose most acclaimed function is to induce the transcription factor T-bet in naive $CD4^+$ T cells thereby directing their differentiation into IFN-γ-producing Th1 cells (Afkarian et al., 2002; Gately et al., 1998; Hsieh et al., 1993; O'Garra et al., 1995). On this account,

it was surprising that CD137L-DCs pre-treated with either C3 or C4 were able to upregulate T-cell production of IFN-γ even though active heterodimeric IL-12p70 was absent (figure 16). Therefore, the presence of R848 (a TLR7/8 agonist) and/or poly (I:C) (a TLR3 agonist) in maturation cocktails C3 and C4, which were documented to greatly enhance IL-12p70 production in classically-derived DCs, failed to promote IL-12p70 production in CD137L-DCs (figure 16), (Boullart et al., 2008).

So how do mature CD137L-DCs propel IFN-γ production in stimulated T cells despite the absence of IL-12p70? This observation is not unheard-of as Schuler et al. previously reported that in a metastatic melanoma clinical trial, IFN-γ-producing $CD4^+$ T cells were detectable even though IL-12p70 secretion by mature classical DCs was miniscule (Schuler, 2010). A possible explanation is that T cells receive pro-Th1 stimulatory signals via a different route; for example by CD70 expressed on APCs (Krause et al., 2009; Soares et al., 2007). Indeed, untreated CD137L-DCs expressed a low amount of CD70 which was highly upregulated upon their maturation using the optimised maturation cocktail which will be discussed in the next section (section 4.4), (figure 37). Expression of CD70 may therefore contribute towards a pro-Th1 immune response.

Alternatively, the production of a different member of the IL-12 cytokine family IL-23, which was upregulated in CD137L-DCs pre-treated with maturation cocktails containing poly (I:C) and/or R848 (C3, C4 or C5), may play a role in the induction of IFN-γ-producing $CD4^+$ T cells (figure 16). This heterodimeric cytokine, which shares a common p40 subunit with IL-12p70, has been described to skew T cells towards an IL-17 producing phenotype dubbed as Th17. However, it was recently shown that IL-23 are able to activate group 1 innate lymphoid cells (ILCs) to co-express IFN-γ together with IL-17 via

downstream IL-23 receptor (IL-23R) signalling (van der Fits et al., 2009). In our experimental context, it is tempting to hypothesise that IL-23 secreted by activated CD137L-DCs may drive IFN-γ synthesis in IL-17 producing T cells, or perhaps also in all IL-23R expressing T cells, thus contributing to the heighten IFN-γ levels observed in the co-culture system (figure 17). In addition, murine data by Codarri et al. showed that IL-23 is also capable of inducing secretion of GM-CSF in activated T cells via the transcription of RORγt (Codarri et al., 2011). Given the importance of GM-CSF in vaccination approaches during subcutaneous tumour growth, IL-23-dependent synthesis of GM-CSF by $CD4^+$ T cells may be another potential mechanism that can contribute to anti-tumour response (van Elsas et al., 1999).

It is worthwhile to note that IL-23 production is also induced by CD137L-DCs activated by LPS plus IFN-γ, although this cocktail does not contribute to the subsequent enhancement of T-cell activation (figures 16 and 17). This may be explained by the fact that in certain cases, IL-23 functions in synergy with other factors such as IL-18 in order to bring about an enhanced IFN-γ production by $CD4^+$ T cells (Lalor et al., 2011). Hence, it is possible to speculate that perhaps R848 plus IFN-γ matured CD137L-DCs, but not LPS plus IFN-γ matured CD137L-DCs, secrete other soluble factors that may work in tandem with IL-23 to potentiate IFN-γ production in T cells.

4.4. A TLR7/8 agonist and IFN-γ are essential and sufficient to functionally mature CD137L-DCs

Maturation cocktails C3 and C4 differ in the presence of poly (I:C), which is only included in the latter (table 4). Since there was no observable biological and functional difference between the CD137L-DCs treated with either

cocktail, we surmised that maturation cocktail C3 contains the components that are key to mediating CD137L-DC activation.

Maturation cocktail C3 consists of five factors namely IL-1β, TNF, IFN-γ, R848 and PGE2. To minimise the need of using large amounts of GMP-grade materials, and to create a simpler protocol for future clinical use, we next aimed to systematically eliminate superfluous components without compromising on functional potency. In the process of generating CD137L-DCs, reverse signalling through CD137L activates the monocytes to produce pro-inflammatory cytokines including IL-1β and TNF (figure 4), (Langstein et al., 1998). These two conceivably autocrine signalling components of C3 together with PGE2 were subsequently removed without any deleterious effects (figures 19-21). Thus, the resultant cocktail containing only R848 and IFN-γ was deduced to be essential and sufficient to mature CD137L-DCs.

CD137L-DCs exposed to LPS plus IFN-γ and R848 plus IFN-γ were able to heighten the expression of maturation markers however, only the latter treatment induced a comparatively more intense T-cell stimulation as compared to untreated CD137L-DCs (figures 15-17 and 19-21). Such an enhanced co-stimulatory activity of CD137L-DCs treated with R848 and IFN-γ is presumably due to a synergistic effect. A possible underlying mechanism might be related to the fact that IFN-γ increases TLR8 expression in human classical DCs (Xu et al., 2006; Zannetti et al., 2010). Considering that R848 is a TLR7/8 agonist, the upregulation of TLR8 by IFN-γ would naturally increase the sensitivity of CD137L-DCs to R848. This agonist has also been reported to trigger immunostimulatory properties in 6-sulfo LacNAc (slan) DCs, a major subpopulation of pro-inflammatory myeloid blood DCs and monocyte-derived DCs (Hackstein et al., 2011; Jahnisch et al., 2013). Both authors describe that DCs pre-treated with R848 had potent T-cell stimulatory potential as evinced

by stronger allogeneic proliferation and IFN-γ release in both $CD4^+$ and $CD8^+$ subsets. It is worth to note that imiquimod, an agonist to TLR7 but not TLR8, failed to deliver a similar outcome (Jahnisch et al., 2013). Thus, it is highly plausible that the functional effects of R848 observed upon maturation of CD137L-DCs are due to agonistic effects solely onto TLR8.

A question of particular interest is why the combination of LPS and IFN-γ does not contribute towards an enhanced potency of CD137L-DCs even though this cocktail has been widely reported to be a potent activator of APCs (Czerniecki et al., 2007; Dohnal et al., 2007). Firstly, we have to understand the biology of CD137L reverse signalling. In the human system, engagement of CD137L leads to activation of the Src family of protein tyrosine kinases and perhaps NF-κB, although there is much debate on the latter due to conflicting published data (Ju et al., 2009; Kwajah and Schwarz, 2010; Sollner et al., 2007). In the murine system however, it has been shown that CD137L physically interacts with TLR4, a receptor for LPS, and associates directly with TLR4 downstream signalling entities consisting of Toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP). This event essentially produces a signalling cascade which is similar to that of the TLR4 pathway (Kang et al., 2007). If such signal transduction also occurs in the human system, then CD137L-DCs would be refractory to the activating effects of LPS via the TIRAP cascade as the engagement of CD137L with CD137-Fc protein would have likely activated this TIRAP-dependent signalling cascade. The production of IL-23 observed upon LPS plus IFN-γ treatment of CD137L-DCs is probably contributed by the alternative signalling cascade by TLR4. Besides the TIRAP-dependent cascade, TLR4 can also activate the TIR-domain-containing adapter-inducing interferon-β / TRIF-related adaptor molecule (TRIF/TRAM) signalling cascade which is not reported to be

associated with CD137L (Kang et al., 2007). This signalling cascade promotes activation of interferon regulatory factor 3 (IRF3), a key transcriptional factor for the IL-23p19 promoter (Smith et al., 2012). Therefore, in CD137L-DCs, the presence of LPS may only contribute to the activation of TRIF/TRAM signalling cascade but not the TIRAP-dependent pathway thereby bringing about only a suboptimal DC activation. On the other hand, R848 is an agonist for endogenous TLR7/8 which has a contrasting downstream signalling pathway compared to TLR4. Signalling through TLR7/8 activates interferon regulatory factor 7 (IRF7) which has been described to induce type-1 interferon and in mice, plays a role in governing induction of $CD8^+$ T-cell responses (Honda et al., 2005).

The triggering of R848-dependent receptors in CD137L-DCs may hence contribute towards greater maturation resulting in a potent enhancement of their biological activities.

4.5. Peptide-pulsed CD137L-DCs potently activate autologous, antigen-specific T cells

Having delineated an optimised maturation cocktail for CD137L-DCs in an allogeneic system, we next strived to investigate the capability of CD137L-DCs as compared to classical DCs, to initiate an antigen-specific response in an autologous setting. To this aim, we utilised a pool of peptides derived from the CMV-encoded protein pp65 as an antigenic stimulus. The advantage of using a peptide pool is that both the $CD4^+$ and $CD8^+$ T-cell population can potentially be activated thus allowing for a more complete characterisation of CD137L-DCs (Zandvliet et al., 2010).

We first tested the capability of CD137L-DCs to activate the pp65 T-cell line within a short period of 18 h. Co-culture within this time frame was insufficient for untreated CD137L-DCs to strongly activate the T-cell line (figure 28). In fact, potency was weaker than even that of the immature classical DCs. On the other hand, brisk restimulation by mature CD137L-DCs enabled $CD4^+$ T cells to potently express IFN-γ to a level higher than mature classical DC. Such enhanced potency of mature CD137L-DCs was not restricted to only the $CD4^+$ T cell subset as a similar trend was also observed in $CD8^+$ T cells (figure 28B). From these data, it was considered that maturation of CD137L-DCs was indeed functional and more importantly, required for the induction of a potent T-cell response.

In the lymph node, however, DC interaction with T cells is dynamic and occurs in three distinct phases (Mempel et al., 2004). This progression from transient serial interaction to the formation of stable contacts with cytokine production and finally rapid T-cell proliferation requires more than 48 h and therefore it was suggested that a longer interaction of CD137L-DCs with pp65 T cells would be physiologically more relevant than an overnight co-culture system.

Longer T-cell restimulation resulted in an altered T-cell activation status. Consistently, the pp65-specific T cells restimulated by autologous CD137L-DCs, be it matured by R848 plus IFN-γ or otherwise, were more activated than those restimulated by mature classical DCs in a 5 day co-culture. This is in contrast to the 18 h co-culture in which only mature CD137L-DCs, but not untreated CD137L-DCs, were effective pp65-specific T-cell activators. The first evidence came about by the examination of T-cell morphology in the co-culture. pp65-specific T cells in the presence of peptide-pulsed CD137L-DCs grew in apparent homotypic aggregates suggesting clonal expansion (figure

30). These aggregates were larger than those formed in the presence of classical DCs signifying greater T-cell growth. Furthermore, by comparing the cytokine profile of IFN-γ, TNF and IL-13, it is clear that CD137L-DCs poise T cells towards activation, and that the restimulation by mature CD137L-DCs further potentiates T-cell activities (figure 31) Nevertheless, quantification of proliferative capacity of these T cells should be resolved in both *in vitro* and *in vivo* conditions in order to determine if CD137L-DCs are capable in expanding functional antigen-specific T cells.

Accumulated findings suggest that regardless of the maturation status, CD137L-DCs are able to activate pp65-specific T cells more potently than mature classical DCs, albeit there is a more robust activation by mature CD137L-DCs. This indicates that untreated CD137L-DCs are already biologically equipped and sufficient to stimulate T cells in an antigen-specific manner while maturation further propels this function. Besides affecting the quality of T-cell activation, maturation also imparts CD137L-DCs with the ability to activate T cells at an earlier time. Maturation of CD137L-DCs prior to co-culture is vital for the induction of a quick response by both the $CD4^+$ and $CD8^+$ antigen-specific T cells but is not entirely necessary if these T cells are restimulated for a longer period (figures 28-31). From these set of data, we conclude that maturation bestows CD137L-DCs with the enhanced capacity to quickly yet effectively activate both $CD4^+$ and $CD8^+$ subsets of antigen-specific T cells although untreated CD137L-DCs themselves are functional APCs.

Examination of the co-culture cytokine profiles suggest that CD137L-DCs support predominantly a Th1 response as evidenced by the multiple-fold increase in both IFN-γ and TNF in the presence of pp65 peptides. Frequently, the levels of Th1 cytokines inversely correlate with those of Th2 cytokines. It

was therefore surprising to detect a profile of IL-13, a cytokine associated with Th2 activation, in response to CD137L-DCs that was similar to that observed for IFN-γ and TNF, albeit to a lower degree in terms of fold change (figure 31). A possible explanation for this profile is that IL-13 may have been upregulated to function as an immunoregulator. Although often labelled as a cytokine mediating allergic inflammation, IL-13 has also been described to dampen inflammatory effects of APCs such as monocytes and macrophages (Doherty et al., 1993; Gordon and Martinez, 2010). In fact, in a viral infection model, Shin et al. concluded that IL-13 is necessary to limit polarised Th1 mediated inflammation, which otherwise would cause severe liver inflammation and pathology (Shin et al., 2007). Thus, IL-13 production may be a negative feedback mechanism as a consequence of the pronounced pro-inflammatory environment that was generated in the presence of CD137L-DCs.

It should be noted that in this assay, bulk supernatants were measured and therefore does not necessarily reflect the exact phenotype of T cells that are highly activated; i.e. $CD4^+$ or $CD8^+$ T cells. For example, high levels of IFN-γ in the supernatant could be contributed by either subset of antigen-specific T cells and the usage of ELISA would not be able to indicate the specific type of T-cell that is predominantly activated. Thus, for improved characterisation of the type of T-cell being activated, it would be beneficial that the application of high resolution read-outs such as by ELISPOT be utilised. Such assays would allow us to enumerate the amount of antigen-specific T cells and also the level of activation based on the amount of cytokines produced per T-cell.

Nevertheless, since CD137L-DCs induced effective production of cytokines associated with $CD4^+$ Th cells, we inferred that perhaps these DCs, on top of their potent activation capacity, are also able to propagate the $CD4^+$ T-cell population. At least in conditions where T cells were restimulated by mature

CD137L-DCs, we noted a shift in the balance of $CD4^+/CD8^+$ T-cell ratio tilting towards the $CD4^+$ population (figure 32). This is in parallel to the observation of a stark increase in expression of MHC class II molecules on the surface of mature CD137L-DCs (figure 24). The subset identities of these $CD4^+$ T cells, however, are yet to be characterised but it is not too far-fetched to suggest that CD137L-DCs are more prone towards a Th1 response. This is evidenced by a multiple fold-increase in Th1 cytokines (IFN-γ and TNF) and in contrast, only a less than 2 fold increase in Th2 cytokine (IL-13) when pp65-specific T cells were restimulated in the presence of peptide antigens (figure 31). Further profiling of the $CD4^+$ T cells by determining the expression of lineage-specific master regulator such as T-bet (Th1-specific), GATA3 (Th2-specific) and RORγT (Th17-specific), and also the expression of other Th cell cytokines such as IL-2, IL-4, IL-5 using ELISPOT assay can be performed to further verify the T-cell subsets that are preferentially activated upon restimulation by CD137L-DCs.

4.6. CD137L-DCs promote a superior killing activity in antigen-specific autologous T cells

Using an allogeneic system, our laboratory previously described the potency of CD137L-DCs in activating $CD8^+$ T cells as evidenced by high perforin and IFN-γ expression (Kwajah and Schwarz, 2010). More recently, we demonstrated that these $CD8^+$ T cells are functionally superior to their counterparts stimulated by mature classical DCs in exerting cytotoxicity against an erythroleukemia cell line (Harfuddin et al., 2013).

In order to further evaluate the immunotherapeutic potential of CD137L-DCs, we addressed their competency to induce anti-tumour functions in an antigen-

specific manner. In line with what we observed in an allogeneic setting, pp65-specific T cells primed by autologous CD137L-DCs, either untreated or mature, had a superior cytotoxic ability as compared to T cells co-cultured with classical DCs. Indeed, T cells stimulated by CD137L-DCs were 2-3 times more potent than T cells primed by classical DCs in inducing the lysis of HLA-matched, antigen-pulsed transformed target cells (figure 33). Remarkably, the difference in T-cell effector potency was miniscule between those stimulated by untreated and mature CD137L-DCs which indicate that CD137L-DCs may actually not require additional exogenous factors to permit potent T-cell activation.

A notable quality of CD137L-DC-activated T cells is their ability to not only instil potent killing of antigen-pulsed targets but also to function effectively with a small number of effector cells as evinced by the strong killing potential at a low E:T ratio. Because the neoplastic microenvironment is often hostile and therefore greatly limits the access of anti-tumour effector T cells, having such highly potent antigen-specific T cells will undoubtedly be beneficial as even a small number of effector cells may be sufficient to control a tumour (Hadrup et al., 2013).

A potential explanation for the enhanced killing activity of CD137L-DCs may be found in the fact that CD137L-DCs stimulate not only $CD8^+$ but also $CD4^+$ T cells, as evidenced by the increase in IFN-γ expression detected in both T-cell populations (figures 28 and 29). $CD4^+$ T cells are essential helper cells that play a role in assisting $CD8^+$ CTLs in their effector activities and also in maintaining them. Indeed, the interactions between $CD4^+$ and $CD8^+$ T cells via CD40 and its ligand (CD40L) results in the downregulation of programmed cell death 1 (PDCD1, an immunosuppressive receptor best known as PD-1) and tumour necrosis factor (ligand) superfamily, member 10 (TNFSF10, an

inducer of apoptosis, best known as TRAIL) on the surface of $CD8^+$ T cells (Bourgeois et al., 2002; Fuse et al., 2009; Janssen et al., 2005). These phenotypic changes dramatically enhance the cytotoxicity of $CD8^+$ T cells and limit their susceptibility to TRAIL-induced apoptosis. Also, soluble factors such as IL-2 that are secreted by $CD4^+$ T cells further act onto $CD8^+$ T cells, thus improving recall responses such as those that occur in the course of lymphocytic choriomeningitis virus (LCMV) infections (Williams et al., 2006).

More recently, the discovery of cytotoxic $CD4^+$ T cells has cast doubt on the dogma that $CD4^+$ T cells merely operate as helpers for $CD8^+$ CTLs. Cytotoxic $CD4^+$ T cells were observed in response to infection or vaccination against viruses such as West-Nile virus, herpes virus, and dengue virus (Brien et al., 2008; Stuller et al., 2010; Yauch et al., 2010). Besides, melanoma-reactive $CD4^+$ T cells have been shown to exert a cytotoxic activity, leading to tumour rejection via MHC class II-restricted interactions (Quezada et al., 2010). These cytotoxic $CD4^+$ T cells appear to exhibit a Th1 phenotype, and the enhanced level of Th1 cytokine IFN-γ by CD137L-DC-primed $CD4^+$ T cells is suggestive of such a cytotoxic activity (figure 28 and 29). It is plausible to infer that perhaps both untreated and mature CD137L-DCs are equipotent in their ability to stimulate $CD8^+$ and $CD4^+$ T cells, and thereby induce an optimal, combined cytotoxic potential in the overall T-cell population. Nevertheless, further investigation of the phenotypic cellular subsets is certainly required in order to verify this possibility.

4.7. Whole protein antigens are not well utilised by CD137L-DCs

The bulk of current DC-based vaccines employ peptides as an antigen of choice (Cintolo et al., 2012). However, this procedure has several shortfalls. Firstly, there are many possible target peptides that can be derived from a

single protein and therefore it is logistically laborious to systematically test all possible peptides for immunogenicity (Gok and Ozcerit, 2012). Secondly, individual immunogenic peptides are specific and will only bind to either MHC class I or class II, partly depending on the number and sequence of amino acid residues. This means that only a single and specific T-cell subtype, either $CD8^+$ or $CD4^+$, will be sensitised at any one point, effectively preventing the development of synergistic T-cell cooperation needed for effective anti-tumour response (Gonzalez-Martin et al., 2011; Takemoto et al., 2010). Thirdly, peptide binding is specific to particular HLA alleles, and hence will only benefit the population expressing that allele. To circumvent these limitations, researchers have devised an alternative strategy by using tumour cell lysates as a source of antigen (Alfaro et al., 2011; Reyes et al., 2013; Wu et al., 2010). TAAs in their native protein form, including as yet undefined ones, are taken-up by the DCs and subsequently processed and presented onto MHC molecules. Thus, a desired immune response should be directed. Major advantages of this procedure include the utilisation of multiple tumour antigens that can be presented on both MHC class I and class II, thereby activating both $CD4^+$ and $CD8^+$ T-cell subsets, and also the presentation on different HLA alleles. Since tumour lysates are essentially a concoction of tumour-associated proteins, we simplified this model by using a native CMV-pp65 protein as a surrogate for tumour lysate.

We clearly demonstrated the dominance of conventional monocyte-derived DCs over both untreated and mature CD137L-DCs in processing and presenting soluble antigens as reaffirmed by the degree of T-cell activation of both subsets (figure 26). This observation may be explained by the lower endocytic capacity of CD137L-DCs than their immature classical counterparts (Ju et al., 2009; Kwajah and Schwarz, 2010). Taking into account the

significance of endocytosis and micropinocytosis in governing the uptake of soluble proteins into APCs, it is likely that CD137L-DCs would have acquired fewer antigens translating to a reduced likelihood of effective antigen presentation (Burgdorf and Kurts, 2008).

Such reduced endocytic capacity is a typical feature of a mature DC phenotype which is suggestive of untreated CD137L-DCs being already relatively mature (Hopkins and Connolly, 2012). Indeed, previously work from our laboratory and current data showed that expression levels of CD83, which is a distinctive marker of mature DCs, hovers in between immature and mature classical DCs, and further activation by R848 and IFN-γ brings about an even further maturation (figure 24B), (Kwajah and Schwarz, 2010). Also, transcriptional profiling analysis, which will be discussed in detail in the next section, concur with this hypothesis as untreated CD137L-DCs are shown to be more closely related to mature classical DCs than immature classical DCs (figures 35B and 35C). CD137L-DCs' character of not possessing any genuine immature form and being already comparatively matured from the onset is a double-edged sword. On the one hand, having a mature phenotype enables CD137L-DCs to be effective T-cell activators as we have demonstrated using CMV pp65 peptides. On the other hand, being already mature would hamper the process of effective engulfment of large quantities of protein for the purpose of antigen acquisition and also prolonged antigen preservation; traits that are unique to the immature forms of DCs (Mellman and Steinman, 2001). This is unlike classical DCs which are endowed with temporal-dependent immature and mature forms; each of which plays crucial roles in the coordinated process of antigen presentation (Hopkins and Connolly, 2012).

In the previous section, it was discussed that peptide-loaded mature CD137L-DCs were competent T-cell activators at both early (18 h) and late (5 days) time-points. However, untreated CD137L-DCs were capable APCs only at the late time-point. Such a similar temporal-dependent event may also occur when protein antigens are use and perhaps CD137L-DCs may be able to instil potent T-cell activation only when the cells are co-cultured for a longer period of time. Nonetheless, such a hypothesis needs to be tested.

In view of the collective data from both peptide-dependent and protein-dependent experiments, we propose that CD137L-DCs are more competent antigen-specific T-cell activators than classical DCs provided that the eventual immunogenic antigen residues are effectively loaded onto MHC molecules. This should be the case for the former assay as exogeneous peptides can directly bind onto the groove of membrane MHCs without the need to undergo internalisation and processing (Blum, 2005). Nevertheless, further investigations are required to identify the most advantageous method for antigen-delivery and presentation. Alternative modes of antigen delivery such as transfection of antigen-coding RNA, DC transduction using viral vectors containing antigen-cassettes, and fusion of DCs with autologous tumour cells can also be looked upon (Koido et al., 2013; Nair et al., 2002; Palucka and Banchereau, 2013).

In addition, we cannot disregard the importance of effective cross-presentation in mounting protective $CD8^+$ T-cell responses, a process critical for anti-tumour immunity (Joffre et al., 2012). Several factors have emerged as important for the modulation of cross-presentation in DCs (Nierkens et al., 2013). This includes the type of antigen encountered and the timing and phase of the immune response. The propensity of CD137L-DCs to undergo this activity has yet to be deciphered, and it is of great interest to further

characterise this feature of CD137L-DCs. Its appreciation will bring us closer to better designed and optimised protocols for future clinical applications.

4.8. *In silico* characterisation of CD137L-DCs

Our aforementioned work on CD137L reverse signalling into monocytes clearly points towards their differentiation into cells with DC-like features. To supplement these *in vitro* information, we conducted gene expression profiling of CD137L-DCs and compared them with the profiles of monocyte-derived DCs (i.e. classical DCs) and macrophages; two cell types known to be derived from peripheral monocytes during inflammation (Geissmann et al., 2010). Essentially, this *in silico* study aimed to identify hitherto unknown functions and/or molecules that are crucial in imparting the biological functions observed in CD137L-DCs. We did not acquire a transcriptional profile for mature CD137L-DCs as these experiments were performed prior to the identification of the optimised maturation factors. However, mature CD137L-DCs were used in subsequent functional assays.

We identified 829 genes which were differentially expressed by CD137L-DCs as a result of CD137 ligand reverse signalling. It is worthwhile to note that any gene expression contributed due the engagement of Fc receptor by the Fc domain of CD137-Fc protein was excluded from our analysis. This was done by eliminating the list of genes expressed by the Fc control monocytes. Thus, the differentially expressed genes of interest are specifically due to signalling via CD137 ligand and its downstream effects.

As anticipated, CD137L-DCs were more closely clustered to classical DCs than any other subsets (figure 35A). In fact, deeper enquiry by cluster distance and cMAP analyses indicated that CD137L-DCs' transcriptional

profile is more similar to mature rather than immature classical DCs (figures 35B and 35C). This reiterates our previous conclusion that described CD137L reverse signalling as a factor driving monocyte differentiation to DCs with a somewhat mature phenotype.

Gene ontology (GO) analysis revealed several biologic categories that are overrepresented upon CD137L reverse signalling in monocytes. Not surprisingly, categories involving immune responses and adhesion were highly enriched (table 11). However, the most enriched category involved regulation of lipids which include biosynthesis as well as metabolic processes of sterols and cholesterols. The implications of lipid regulation in CD137L-DC development are still unknown and studies are currently being engaged in our laboratory.

To decipher the unique qualities that make CD137L-DCs more potent than classical DCs, we derived a set of genes which were highly upregulated by CD137L-DCs with respect to mature classical DCs (tables 8 and 11). GO analysis revealed categories involved in adhesion processes, locomotion behaviour and immune responses to be the most enriched. As CD137L-DCs are adherent cells, it was expected that transcription of adhesion-associated genes would be upregulated (figure 24A). This said, the discovery of this process being highly significant in CD137L-DCs as compared to mature classical DCs is novel. Functional studies comparing CD137L-DCs with classical DCs correlated with GO analysis since the former were much more prone to attachment than the latter upon being re-cultured (figure 38). With this observation, we deduce that the adhesion processes may possibly be an important contributing factor that confers CD137L-DCs with a greater T-cell activation capacity.

A probable benefit of possessing such adhesion properties is the ability to form strong cell-cell interaction via stable immune synapses. Although this hypothesis has not been tested in CD137L-DCs, DC-associated adhesion molecules such as ICAM-1, LFA-3 and several integrins are prerequisites for supramolecular activating complex (SMAC) formation, essential for the initiation of the immune synapse with T cells (Rodriguez-Fernandez et al., 2010; Sims and Dustin, 2002). In fact, ICAM-1-deficient DCs did not form long-lasting interaction with $CD8^+$ T cells which resulted in inadequate CTL priming and therefore reduced production of IFN-γ both *in vitro* and *in vivo* situations (Scholer et al., 2008).

Surface expression of ICAM-1 was indeed detected on CD137L-DCs and was further increased upon their maturation (figure 37B). Yet mature classical DCs expressed more ICAM-1 suggesting that this molecule is not a main factor contributing to CD137L-DCs' superior ability to activate T cells. Two other molecules CLEC5A and PDPN which are associated with viral attachment and cellular migration, respectively, are likewise highly expressed by CD137L-DCs (figure 37), (Acton et al., 2012; Chen et al., 2008). Interestingly, both molecules are also expressed on macrophages but not classical DCs implying that CD137L-DCs are phenotypically unique DCs. Efforts to study the impact of CLEC5A and PDPN on T-cell activation by CD137L-DCs were, however, hampered due to the lack of commercially available antagonists.

The adhesion molecule, ALCAM also known as CD166, was found to be highly upregulated in CD137L-DCs and classical DCs although it is more prominent in the former (figure 37). Several studies have described ALCAM interaction with CD6 on T cells to be essential for immunological synapse stabilisation and optimal T-cell activation and proliferation (Gimferrer et al., 2004; Hassan et al., 2004; Zimmerman et al., 2006). Our studies

demonstrated that the blocking of ALCAM on CD137L-DCs, but not on classical DCs, reduced their capacity to activate T cells as evidence by the reduction in T-cell proliferation (figure 40). Interestingly, while the effect of ALCAM blockade is more prominent on mature CD137L-DCs rather than untreated CD137L-DCs, expression of ALCAM is essentially similar between these two populations (figure 37). An obvious question would be why ALCAM appears to be more crucial for mature CD137L-DCs rather than untreated CD137L-DCs? This might be explained by an observation by Zimmerman and colleagues which illustrates a more robust binding of ALCAM to its ligand in mature rather than immature DCs, possibly due to avidity regulation; a process where molecules are redistributed into clusters on the cell membrane (Nelissen et al., 2000; Zimmerman et al., 2006). Thus, the biological contribution of ALCAM is naturally more impactful in mature DCs and any functional abrogation will result in a more severe consequence. In our hands, blocking of ALCAM on classical DCs did not impose any inhibitory effects on their potential to activate T cells suggesting that ALCAM function is dispensable for classical DCs. Collectively, we surmise the significance of ALCAM as a valuable contributor in augmenting CD137L-DCs' biological capacity to stimulate T cells.

Once a stable immune synapse is formed, MHC-TCR interactions as well as co-stimulatory interactions dictate the quality of the resulting T-cell response (Thauland and Parker, 2010). Hence, we studied the co-stimulatory molecules that may be necessary to confer a T-cell activating potency in CD137L-DCs. Transcription profiling data suggest that OX40L and CD70, which are respective ligands for OX40 and CD27 found on T cells, may be such co-stimulatory molecules (figure 37A). OX40L protein was not detected but CD70 was found to be expressed on the surface membrane of CD137L-

DCs although this only occurred upon their maturation implying that CD70 may have been pre-synthesised and stored in intracellular compartments prior to maturation (figure 37B). Moreover, CD70 is delivered to the immune synapse by shared intracellular trafficking with MHC class II; a molecule which is only highly expressed by CD137L-DCs as a consequence of maturation (figure 24B), (Keller et al., 2007). CD70 is a member of the TNFRSF and its upregulation by DCs is a crucial component of CD40-mediated licensing since blockade of CD70 abrogates pro-stimulatory effects by CD40 (Taraban et al., 2004). CD70 also directly stimulates T cells by interacting with T-cell-bound CD27 which is required for $CD8^+$ T-cell priming and also maintaining their survival (Hendriks et al., 2003; Keller et al., 2008). Albeit not unique in mature CD137L-DCs, as a similar CD70 expression is observed in mature classical DCs, it is reasonable to speculate that the likelihood of stronger immune synapse formation by mature CD137L-DCs will provide a platform for a more sustained CD70-CD27 interaction which is essential for priming of T cells (Keller et al., 2007; Keller et al., 2008).

Another membrane-bound molecule which is associated with mature DCs is CD83 (Lechmann et al., 2002). Ample lines of evidence have suggested the significance of human CD83 as a co-stimulator for the induction of stronger T-cell activation including naive $CD8^+$ T cells, although this is not the case in the murine system. (Hirano et al., 2006; Kretschmer et al., 2008; Prazma et al., 2007; Prechtel et al., 2007; Scholler et al., 2001). Expression of CD83 on CD137L-DCs has been previously reported by our laboratory and this was one of the early indications denoting that CD137L reverse signalling in monocytes led to differentiation of a DC-like phenotype (Kwajah and Schwarz, 2010). Intriguingly, transcriptional expression data did not point towards a high CD83 expression on untreated CD137L-DCs although flow

cytometric analysis proved otherwise (figure 37). Such seemingly contradictory observations can be explained by the fact that CD83 is actually preformed and stored in perinuclear regions of monocytes and immature DCs, and only gets stably expressed on the cell surface upon maturation such as by LPS (Cao et al., 2005). Thus, CD137L reverse signalling in monocytes may induce events that drive the trafficking of intracellular CD83 onto the cell membrane rather than initiating CD83 gene transcription. This event is likely to be potentiated by pre-treatment of CD137L-DCs with R848 and IFN-γ resulting in the heightened surface protein expression (figure 37B). Just like with CD70, CD83 is not unique in CD137L-DCs and the proposed stronger immune synapse formation may be the deciding factor that drives potent T-cell responses. The abovementioned co-stimulatory molecules are nonetheless vital players in the overall commitment to mount an effective immune response.

4.9. CD137L-DCs: Towards their use as therapeutic cancer vaccines

These encouraging *in vitro* findings pave the way for future requisite work focused on developing this novel form of DCs for their use in anti-tumour immunotherapy. Various key features make CD137L-DCs an attractive alternative to the current protocols for DC development. These include (1) the requirement of fewer exogenous proteins and cytokines for DC generation, (2) the ability to potently stimulate antigen-specific effector T cells of high cytotoxic potential and (3) the capacity to activate IFN-γ-producing $CD4^+$ Th cells. Significantly, our novel form of DC is functionally more potent than classically-generated DCs in activating antigen-specific T cells and this warrants the need to look upon developing CD137L-DC-base vaccine protocols. As the ultimate goal is to use CD137L-DCs in DC-based

immunotherapy, further characterisation of their development and potential *in vivo* effects is required.

4.9.1. Production and *in vivo* character of CD137L-DCs

An advantage of producing CD137L-DCs is that they only require CD137-Fc protein as a prerequisite, making it the simplest protocol for DC generation. On the other hand, the current protocol requires two exogenous factors, namely GM-CSF and IL-4, although it was recently described that GM-CSF is actually dispensable for *in vivo* differentiation of inflammatory DCs (Greter et al., 2012). This provides evidence of the possibility of alternative methods of DC generation. Also, several additional factors (generally ranging from 4-6 factors) are then needed to ensure maturation thus inflating production costs (Erdmann and Schuler-Thurner, 2010). The simplicity of developing CD137L-DCs may be an effective strategy to reduce overall costs of therapy.

Our current procedure for CD137L-DC generation requires CD137-Fc proteins to be coated onto culture plates before freshly isolated monocytes are seeded. This action allows cross-linking of homotrimeric CD137L and the subsequent downstream reverse signalling to be initiated; a process which cannot occur if CD137-Fc proteins are added solubly. Such stringent requirement for cross-linking would curb the production of CD137L-DCs using commonly used cell culture bags. Fortunately, alternative GMP-grade close-system such as the Cell Factory is available and can be used for propagation of adherent cells (Erdmann and Schuler-Thurner, 2010). We also showed that the generation of CD137L-DCs is comparable to the protocol of classical DCs in terms of the final quantity of DCs acquired, and therefore does not impede the process of large-scale production (figure 24C).

As earlier presented, maturation of CD137L-DCs is required for early antigen-specific T-cell activation but is dispensable if T cells are stimulated for a longer period. Whether or not DC maturation prior to infusion is a necessity for *in vivo* initiation of an anti-tumour response has yet to be determined. Nevertheless, maturing CD137L-DCs may boost their tendency to migrate to the lymph nodes and induce competent T-cell activation due to the increase in CCR7 expression (figure 15). The migratory potential of CD137L-DCs is currently being explored in the laboratory via *in vitro* techniques such as the utilisation of Boyden chambers (trans-chambers).

Additionally, the ability to generate CD137L-DCs from cancer patient-derived peripheral monocytes should be attempted especially since monocytes from such individuals are often functionally aberrant as described in introduction (section 1.1.1.3).

4.9.2. Improving antigen-loading and processing in CD137L-DCs

A limitation of CD137L-DCs is their inefficiency to utilise whole protein antigens which poses a huge technical drawback. Confining to peptides alone would benefit only an HLA-specific population and this is inadequate since the goal is to expand the use of CD137L-DCs as a first approach for anti-tumour therapy.

To circumvent this issue, several alternatives can be explored including the transfection of antigen-coding RNA and fusion of DCs with autologous tumour cells (Koido et al., 2013; Nair et al., 2002). Another approach that combines CD137L-DC development with antigen loading can also be used in our context. Here, CD137-Fc proteins can be physically conjugated with protein antigens to create a complex which is subsequently used to differentiate

monocytes into CD137L-DCs. Engagement of this complex with CD137L will activate monocytes towards CD137L-DC differentiation, and subsequently the complex will be internalised by endocytosis; a process that occurs in the natural CD137-CD137L system (Ho et al., 2013). This mechanism may assist CD137L-DCs with antigen uptake allowing for improved antigen processing and presentation onto MHC molecules which we reckon will beget greater T-cell activation. We are, in the laboratory, currently studying this approach and it is hoped that it will be a feasible option for the loading of antigens into CD137L-DCs.

4.9.3. Quality of vaccine-induced T cells

Cancer immunotherapy has focused primarily on engaging tumour-reactive $CD8^+$ CTLs with the capacity to directly kill tumour cells and also differentiate into long-lived memory cells (Dudley et al., 2002; Palucka and Banchereau, 2013). Recently, specific phenotypes of $CD8^+$ T cells with superior anti-tumour effects have been defined. For example, $CD8^+$ T cells expressing CD103 ($\alpha E\beta 7$) integrin facilitates their adherence to tumour expressing E-cadherin, bringing about their demise and eventual rejection (Le Floc'h et al., 2007). Also, $CD8^+$ T cells with characteristics of central memory cells, i.e. $CCR7^+$, $CD27^+$, $CD28^+$ and $CD62L^+$, have more profound anti-tumour effects than highly differentiated cells that have lost these makers (Gattinoni et al., 2005). Such detailed phenotyping of CD137L-DC-stimulated T cells have not been performed and should be looked into.

The importance of tumour-specific $CD4^+$ T cells cannot be ignored as they play critical supporting roles for $CD8^+$ T cells, and are by themselves able to evoke direct cytotoxicity (Quezada et al., 2010). CD137L-DCs are able to promote $CD4^+$ T cells to produce high levels of IFN-γ, a cytokine signifying a

Th1 response. However, the phenotype status remains to be investigated in detail, especially considering that IL-13, a prototypic Th2 cytokine, was found to be secreted by CD137L-DC-primed T cells. Whether or not CD137L-DCs also activate cytotoxic $CD4^+$ T cells is unknown.

An undesirable feature that may compromise anti-tumour effects is T-cell exhaustion. Although CD137L-DC stimulated T cells are able to secrete high amounts of IFN-γ and to induce effective antigen-specific killing of target cells, their ability to undergo additional rounds of re-stimulation has not be defined. It is paramount that T cells primed by CD137L-DCs are not exhausted and are able to mount effective anti-tumour reactions. This is especially so since T cells found in the vicinity of tumours often display higher markers of T-cell exhaustion such as PD-1 and CTLA-4 than T cells in blood or normal adjacent tissues (Ahmadzadeh et al., 2009; Wang et al., 2012).

Such T-cell features should be ascertained before attempting to embark on clinical trials.

4.9.4. Additional future work

In this project, we employed CMV-derived pp65 peptides as a model antigen to describe the antigen-specific T-cell activation ability by CD137L-DCs. A further advance and a logical next step to develop CD137L-DCs for clinical use would be testing whether CD137L-DCs can also induce potent immune responses against human tumours. A candidate antigen of choice is MART-1/MelanA, an immunogenic melanosomal-derived antigen expressed in more than 90 % of melanomas (Coulie et al., 1994; Kawakami et al., 1994). Highly immunogenic modified short peptides with optimised HLA anchor residues can be loaded onto CD137L-DCs to test for their ability to activate autologous

MART-1/MelanA-specific T cells, and can also be followed-up with cytotoxic assays. Many TAAs are self-derived, therefore, the T cells that are directed towards TAAs are often in a naive state. Accordingly, determining the ability of CD137L-DCs to prime naive, tumour-specific T cells is of upmost importance and can be performed using the MART-1/MelanA system (Chauvin et al., 2012). Other potential antigens that should be tested are the EBV-derived LMP1 and LMP2A that are commonly detected in nasopharyngeal carcinoma (NPC) samples (Lo et al., 2004; Young and Murray, 2003). T-cell clones specific to LMP1 and/or LMP2A can be generated and the capacity of CD137L-DCs to restimulate these clones in an antigen-specific manner should be performed.

Gene expression profiling analysis identifies CD137L-DCs as being transcriptionally similar to classical DCs (figure 35). Yet, several macrophage-like features such as high PDPN and CLEC5A are also prominent in these cells (figure 37). Further characterisation to ascertain whether such molecules are implicated in conferring T-cell activation ability may also be looked upon to better understand the activities of CD137L-DCs. Thorough scrutiny of the vast microarray data may also hold crucial clues that may help to decipher the mechanisms that define the potency of CD137L-DCs. For example, inhibitory PD-L1 and PD-L2 which bind to PD-1 on T cells to attenuate immune responses are transcriptionally highly expressed in mature classical DCs but not in CD137L-DCs (appendix I), (Keir et al., 2007). The absence of this inhibitory mechanism may explain why CD137L-DC-primed T cells become more activated with time (figure 31). Thus, a more detailed investigation of the transcriptional profile of CD137L-DCs should be performed.

4.10. Conclusion

Immunotherapeutics are currently used primarily to supplement traditional treatment approaches in anti-cancer regimes. With more robust and vigorous biologic therapy protocols in the pipeline, we predict a potential shift in clinical acceptance for cancer vaccines from a complementary procedure to a first-line treatment.

To aid in this vision, we have discovered a novel method of generating DCs with superior ability to activate both $CD4^+$ Th cells and $CD8^+$ CTLs in an antigen-specific manner using pulsed peptides as a source of antigen. More importantly, the resultant T cells are exceptional in inducing the killing of peptide-loaded target cell line thus displaying properties that are desired of an anti-tumour response. Through *in silico* analysis, we were able to show that CD137L-DCs are transcriptionally similar to classical DCs but are unique in their own right as they express several shared molecules defining both DCs and macrophages. In combination with functional analysis, ALCAM was identified as a possible contributor towards CD137L-DCs' biological functions though it is likely that many other yet unknown factors are also involved.

This current study presents the unparallel prowess of CD137L-DCs as potent APCs in a viral model, and it is our desire to apply these DCs onto a tumour setting. Granted, CD137L-DCs may yet be developed into a 'next-generation' DC-based anti-tumour vaccine capable of advancing medical treatment for cancer.

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APPENDICES

Appendix I: Supplementary data

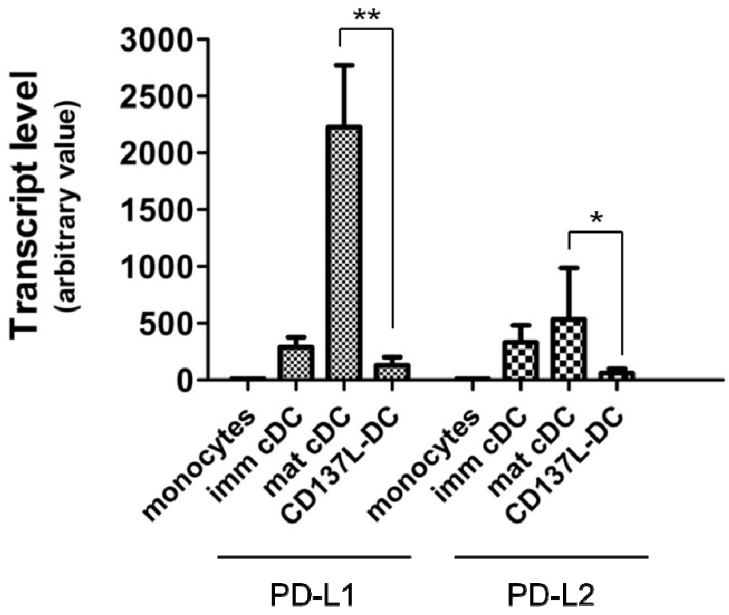

Figure A1: Transcriptional profile of PD-L1 and PD-L2 on various DCs/APCs.

Appendix II: Gene lists for DAVID analysis

Table A1: CD137L-DC DEG which are ≥ 2x fold-change compared to monocytes

Probe_ID	Gene_Symbol	Probe_ID	Gene_Symbol
ILMN_2160476	CCL22	ILMN_2092441	LRP12
ILMN_2374449	SPP1	ILMN_1656904	SLC1A4
ILMN_1651354	SPP1	ILMN_1691539	LAT
ILMN_2192072	MMP7	ILMN_1701195	PLA2G7
ILMN_1801205	GPNMB	ILMN_1709043	C9orf46
ILMN_1752562	CXCL5	ILMN_1806434	PAQR5
ILMN_2170814	LAMP3	ILMN_2407824	ATP1B1
ILMN_2413644	TM4SF19	ILMN_2089329	SPRY2
ILMN_1685403	MMP7	ILMN_1730291	ATP1B1
ILMN_1656310	INDO	ILMN_2188862	GDF15
ILMN_2171384	CXCL5	ILMN_1758057	TOR1AIP2
ILMN_2407389	GPNMB	ILMN_1704985	CYP27A1
ILMN_1793730	TM7SF4	ILMN_2199439	CA2
ILMN_1740418	CYP27B1	ILMN_1670325	SLC47A1
ILMN_2065773	SCG5	ILMN_2081883	IQCK
ILMN_1786444	LPL	ILMN_1701483	SYP
ILMN_2289593	FXYD2	ILMN_1726230	CD1B
ILMN_1710186	CCL17	ILMN_1700690	VAT1
ILMN_3239965	IDO1	ILMN_1721623	APOO
ILMN_1773389	PLTP	ILMN_1803647	FAM162A
ILMN_2087656	SLCO2B1	ILMN_1786469	FBXO22
ILMN_1794803	NDP	ILMN_3277905	LOC645313
ILMN_1867119		ILMN_1758827	RTN4IP1
ILMN_1715131	CCR7	ILMN_1725518	ANGPTL6
ILMN_1808325	TM4SF19	ILMN_2408796	C19orf28
ILMN_1670490	PDPN	ILMN_2102330	COL8A2
ILMN_1725510	DHCR24	ILMN_1743620	RARRES1
ILMN_1802653	EBI3	ILMN_2077905	PTGFRN
ILMN_1705750	TGM2	ILMN_1806249	IL1RN
ILMN_3247139	C17orf96	ILMN_1674050	COL8A2
ILMN_2316236	HOPX	ILMN_1662795	CA2
ILMN_1789502	GPC4	ILMN_1659801	ATP6V1C1
ILMN_1754538	C10orf58	ILMN_1782635	YARS2
ILMN_2124221	CLLU1OS	ILMN_1682781	TEAD2
ILMN_1761199	SLCO2B1	ILMN_1772964	CCL8
ILMN_2223903	PPIC	ILMN_1691339	CLEC1A
ILMN_1689842	SC4MOL	ILMN_1742853	LOC652184
ILMN_1688480	CCND1	ILMN_1708743	NT5DC2
ILMN_1690170	CRABP2	ILMN_1708041	PLEKHF1
ILMN_1669032	PPIC	ILMN_1683273	SNAPC5
ILMN_2319000	MATK	ILMN_1786108	TUT1

ILMN_3250257	ACVRL1	ILMN_1810267	DNHD1
ILMN_1767113	AOX1	ILMN_1762713	C19orf59
ILMN_2329927	ABCG1	ILMN_1851547	
ILMN_1785380	SLC1A2	ILMN_1783712	LOC400506
ILMN_1787657	CLDN12	ILMN_1724699	ACAD8
ILMN_1748538	ALDH1A2	ILMN_1804568	HOMER1
ILMN_1704369	LIMA1	ILMN_1740875	FPR2
ILMN_1843198		ILMN_3300358	ZNF84
ILMN_1814022	NR1H3	ILMN_1774806	SLC9A7
ILMN_1765557	OLFML2B	ILMN_1664718	CYP51A1
ILMN_1784553	SDC2	ILMN_1693664	POMGNT1
ILMN_2317923	TMEM132A	ILMN_2134974	RAB38
ILMN_1694400	MSR1	ILMN_1788810	C12orf30
ILMN_1705849	SPR	ILMN_2085236	SNX24
ILMN_1720889	SC4MOL	ILMN_1738147	NES
ILMN_2227248	SLAMF9	ILMN_2128770	CDR2L
ILMN_2413779	SEZ6L2	ILMN_2406410	RHBDD2
ILMN_3307791	FAM69A	ILMN_1737561	LOC88523
ILMN_1704376	GLDN	ILMN_1769383	GIMAP5
ILMN_1784532	COL22A1	ILMN_1751898	C12orf4
ILMN_1717477	PSD3	ILMN_1769926	DBN1
ILMN_1659106	PHLDA3	ILMN_2225595	ACAD8
ILMN_2165867	DHCR7	ILMN_1743199	EGR2
ILMN_1689329	SCD	ILMN_1724946	SPINT1
ILMN_1738578	FILIP1L	ILMN_1702168	HSD17B12
ILMN_1704753	EPAS1	ILMN_1758895	CTSK
ILMN_2365307	CD276	ILMN_1680805	IL28RA
ILMN_1696302	FABP5	ILMN_2049766	NFE2L3
ILMN_2093027	MYO1B	ILMN_1770911	NRSN2
ILMN_1797822	SEL1L3	ILMN_1796335	LPCAT2
ILMN_1717262	PROCR	ILMN_1751328	FAM83H
ILMN_1794782	ABCG1	ILMN_1781819	PAPSS1
ILMN_2188722	GLS	ILMN_3236135	FAM86D
ILMN_1761247	PIR	ILMN_2100287	CCDC147
ILMN_2212909	MELK	ILMN_3210914	LOC344887
ILMN_1785170	ARMCX2	ILMN_3197097	TSTD1
ILMN_1770338	TM4SF1	ILMN_2099277	HTRA4
ILMN_2398572	FXYD2	ILMN_1670322	FCHO2
ILMN_3245458	SNORA61	ILMN_1751228	C20orf46
ILMN_1808404	RHBDF1	ILMN_3235853	S1PR1
ILMN_1763638	BCAR3	ILMN_1805916	NIPSNAP1
ILMN_1780831	SLC6A12	ILMN_1783909	COL6A2
ILMN_2380163	PTPRF	ILMN_2095133	SPTAN1
ILMN_1732151	COL6A1	ILMN_1764861	ISOC1
ILMN_1690040	TM7SF2	ILMN_1756992	MUC1
ILMN_1809483	HSD17B14	ILMN_1729167	EFTUD1

ILMN_1669362	IGFBP6	ILMN_3193306	C14orf109
ILMN_1805807	SLC30A3	ILMN_1715068	AQP9
ILMN_1666546	DUSP14	ILMN_1809437	RHBDD2
ILMN_2383707	ALDH1A2	ILMN_1799024	VAC14
ILMN_3246538	LOC100133866	ILMN_1786273	C1orf122
ILMN_1800091	RARRES1	ILMN_2051867	PTCD2
ILMN_1911721		ILMN_1699139	CYP19A1
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ILMN_1739001	TACSTD2	ILMN_2119774	CYP2R1
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ILMN_1764723	SH3PXD2B	ILMN_1785405	SLC17A9
ILMN_1735157	GALNT12	ILMN_2121816	GPR137B
ILMN_1779257	CD40	ILMN_1660031	P2RY6
ILMN_1744381	SERPINE1	ILMN_1795507	ABCA6
ILMN_1815190	METTL1	ILMN_1794386	IL2RG
ILMN_1752965	GREM1	ILMN_1666665	COL23A1
ILMN_2374683	PTPN13	ILMN_1666902	GPR114
ILMN_3178258	FABP5L2	ILMN_1714082	CMAS
ILMN_1678841	UBD	ILMN_1673892	GK5
ILMN_3306997	METTL1	ILMN_1796508	TOP1MT
ILMN_1739222	ETV5	ILMN_1813338	LAG3
ILMN_3210741	LOC642956	ILMN_2183216	TTRAP
ILMN_2405324	IL28RA	ILMN_1745152	UQCC
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ILMN_1726624	YPEL4	ILMN_1862521	
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ILMN_1726981	VEGFB	ILMN_1781626	C1S
ILMN_1741632	RAB3IL1	ILMN_1731783	ATP1A1
ILMN_1682099	TNFAIP8L3	ILMN_3179620	LOC100129673
ILMN_1663538	CLYBL	ILMN_1741727	QPCT
ILMN_1741021	CH25H	ILMN_1725314	GBP3
ILMN_1717727	LOC284293	ILMN_1680727	GLRX2
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ILMN_1804735	CBS	ILMN_1747759	WSB1
ILMN_1914249		ILMN_1915188	

ILMN_1809859	PCGF2	ILMN_1794825	ALDH3A2
ILMN_1913060		ILMN_2229261	SIGLEC16
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ILMN_1815690	TIE1	ILMN_3209232	LOC644496
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ILMN_1765459	S100A13	ILMN_1698243	C1orf85
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ILMN_1793476	PRKCDBP	ILMN_2362974	CASP7
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ILMN_1702501	RPS6KA2	ILMN_1703487	LMO4
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ILMN_1728581	C3orf18	ILMN_1678353	FARP1
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ILMN_1758750	EARS2	ILMN_1705297	MYBPH
ILMN_1656378	NMT2	ILMN_1688089	PEBP1
ILMN_1655100	LOC644680	ILMN_1738010	CTNS
ILMN_1668283	HYAL2	ILMN_1680781	C14orf135
ILMN_1718972	MFSD3	ILMN_1699772	RRAGD
ILMN_1696031	C15orf21	ILMN_1770020	PPIL3
ILMN_1654411	CCL18	ILMN_1659888	PPP1R14B
ILMN_2131381	PDE3B	ILMN_1690099	ITGB1BP1
ILMN_3298511	LOC728537	ILMN_1663512	COX5B
ILMN_1770466	ATP5G3	ILMN_3219631	LOC647081
ILMN_1749792	SORBS1	ILMN_2106167	RAP1GDS1
ILMN_1697220	NT5E	ILMN_2413780	SEZ6L2
ILMN_1724789	CD59	ILMN_1736103	ITPR2
ILMN_1885771		ILMN_1807515	CSTF2T
ILMN_1771261	SYNC1	ILMN_2372136	P4HTM
ILMN_3234089	N4BP2L2	ILMN_1761031	PTPDC1
ILMN_1797585	MYO1B	ILMN_3236928	ROBLD3
ILMN_2383349	STEAP3	ILMN_1726306	HMBS
ILMN_1685703	ACOX2	ILMN_2245305	ABHD12
ILMN_1700047	ALAS1	ILMN_1791306	C9orf103
ILMN_1702231	C1orf54	ILMN_1803392	TAX1BP3
ILMN_1774739	MMP14	ILMN_1712291	MICALL2
ILMN_1677814	ABCC3	ILMN_2208495	LASS5
ILMN_2338963	SLC29A1	ILMN_3243705	PDXDC1
ILMN_2148913	TMEM45A	ILMN_1664761	TMEM138
ILMN_2404625	LAT		

Table A2: CD137L-DC DEG which are ≥ 2x fold-change compared to mature cDCs

Probe_ID	Gene_Symbol	Probe_ID	Gene_Symbol
ILMN_1752562	CXCL5	ILMN_1731745	NINJ2
ILMN_2171384	CXCL5	ILMN_2413064	ST6GALNAC4
ILMN_1780465	CLEC5A	ILMN_2224103	PAPSS1
ILMN_2065773	SCG5	ILMN_1756572	COQ2
ILMN_1699695	TNFRSF21	ILMN_1674050	COL8A2
ILMN_1762713	C19orf59	ILMN_1742547	NRP1

ILMN_1767113	AOX1	ILMN_1808354	SLC4A7
ILMN_1789502	GPC4	ILMN_1781867	FOXD4L1
ILMN_1769299	MTMR11	ILMN_1767253	RRP12
ILMN_2361603	NDRG2	ILMN_3251155	PCBP2
ILMN_2413779	SEZ6L2	ILMN_1732151	COL6A1
ILMN_1789196	TPM2	ILMN_1666546	DUSP14
ILMN_1669523	FOS	ILMN_1805371	HNRPM
ILMN_1769926	DBN1	ILMN_2347592	NMB
ILMN_2380163	PTPRF	ILMN_1781819	PAPSS1
ILMN_2199439	CA2	ILMN_1725518	ANGPTL6
ILMN_2093027	MYO1B	ILMN_1735231	ZSWIM4
ILMN_1757604	TPM2	ILMN_1758806	C21orf2
ILMN_1670638	PITPNC1	ILMN_2102330	COL8A2
ILMN_2316236	HOPX	ILMN_1680364	CD109
ILMN_1705686	NRGN	ILMN_1660806	CSRP2
ILMN_1739001	TACSTD2	ILMN_1689160	DPEP2
ILMN_1670535	NDRG2	ILMN_1662390	ASPHD1
ILMN_1752965	GREM1	ILMN_1800412	BMP1
ILMN_2374683	PTPN13	ILMN_1807177	KIAA1797
ILMN_1669362	IGFBP6	ILMN_1785380	SLC1A2
ILMN_1686555	FYN	ILMN_3205353	LOC100131294
ILMN_1784532	COL22A1	ILMN_1682015	GAL
ILMN_1670490	PDPN	ILMN_2150851	SERPINB2
ILMN_1662795	CA2	ILMN_3211302	LOC646909
ILMN_1658926	NOTCH3	ILMN_1677814	ABCC3
ILMN_1685703	ACOX2	ILMN_1682099	TNFAIP8L3
ILMN_1704376	GLDN	ILMN_2406410	RHBDD2
ILMN_1815057	PDGFRB	ILMN_1722981	TLR5
ILMN_1784948	SPOCD1	ILMN_2188722	GLS
ILMN_3248591	LTBP2	ILMN_1809437	RHBDD2
ILMN_2205963	C10orf54	ILMN_1728107	GNG7
ILMN_1745623	EFCAB4A	ILMN_1786326	KCTD15
ILMN_1728799	FBP1	ILMN_1805930	CSF1
ILMN_1690170	CRABP2	ILMN_1743275	SH3RF3
ILMN_1653766	CCL24	ILMN_1718972	MFSD3
ILMN_1805807	SLC30A3	ILMN_1693552	CD300A
ILMN_2100437	HBB	ILMN_1717262	PROCR
ILMN_2229261	SIGLEC16	ILMN_1718621	TSPAN32
ILMN_3245630	ECSCR	ILMN_2124221	CLLU1OS
ILMN_1687857	ST6GALNAC4	ILMN_2086105	SPRY4
ILMN_1765274	CAPN11	ILMN_3236130	LOC100132547
ILMN_2340347	PC	ILMN_1781791	PRRG1
ILMN_1779965	AK1	ILMN_1724612	SLC25A35
ILMN_1715885	PTPN22	ILMN_1751228	C20orf46
ILMN_2227248	SLAMF9	ILMN_1722738	ROGDI
ILMN_2068104	TFPI2	ILMN_1716006	C18orf54

ILMN_1680110	C10orf116	ILMN_3239500	FAM135B
ILMN_1731374	CPE	ILMN_1678423	SPA17
ILMN_1914249		ILMN_1784005	LOC644615
ILMN_2064725	METTL7B	ILMN_2131381	PDE3B
ILMN_1769615	FLRT2	ILMN_2289849	FCGR2A
ILMN_2101278	RGS18	ILMN_1736127	KIAA0773
ILMN_1677441	C20orf123	ILMN_3248352	CCDC88C
ILMN_2374687	PTPN13	ILMN_1785170	ARMCX2
ILMN_1702501	RPS6KA2	ILMN_1670870	ALCAM
ILMN_1750181	TESC	ILMN_1659888	PPP1R14B
ILMN_1808226	RGS16	ILMN_1751901	TMEM163
ILMN_3251672	DSG2	ILMN_3245458	SNORA61
ILMN_1815690	TIE1	ILMN_1737089	CAPN5
ILMN_1662619	TFPI	ILMN_2077905	PTGFRN
ILMN_2329927	ABCG1	ILMN_1770338	TM4SF1
ILMN_2380801	FYN	ILMN_1704753	EPAS1
ILMN_1762899	EGR1	ILMN_3210171	LOC389156
ILMN_2089329	SPRY2	ILMN_1666902	GPR114
ILMN_1811736	ENTHD1	ILMN_1665571	LOC644869
ILMN_1708743	NT5DC2	ILMN_1890614	
ILMN_1698666	CST6	ILMN_2383349	STEAP3
ILMN_1707124	TFPI	ILMN_2119774	CYP2R1
ILMN_1811470	PLEK2	ILMN_1666932	FCGR2A
ILMN_2124585	GREM1	ILMN_2148469	RASL11B
ILMN_1726624	YPEL4	ILMN_1701643	GDPD5
ILMN_1806434	PAQR5	ILMN_1804568	HOMER1
ILMN_2289593	FXYD2	ILMN_1709795	RAC2
ILMN_1747673	RASL10A	ILMN_1801043	GSN
ILMN_2316386	GPBAR1	ILMN_1712352	DOCK3
ILMN_1711439	EMILIN1	ILMN_1770922	TMEM45A
ILMN_1675191	GAPT	ILMN_1736704	DIXDC1
ILMN_1781207	FYN	ILMN_1752159	AHNAK
ILMN_2143685	CLDN7	ILMN_3285410	LOC642738
ILMN_2319000	MATK	ILMN_1775257	PROK2
ILMN_1810996	COL24A1	ILMN_1655614	DSP
ILMN_1697220	NT5E	ILMN_1726306	HMBS
ILMN_1794782	ABCG1	ILMN_1697115	FLJ12355
ILMN_1704985	CYP27A1	ILMN_1691736	ST6GALNAC6
ILMN_1809859	PCGF2	ILMN_2165473	MID1IP1
ILMN_1659106	PHLDA3	ILMN_1792495	AHNAK
ILMN_1898723		ILMN_1653026	PLAC8
ILMN_1724699	ACAD8	ILMN_2357577	PRKAA1
ILMN_3298511	LOC728537	ILMN_1745130	RBM9
ILMN_3242271	GAPT	ILMN_1758827	RTN4IP1
ILMN_1783996	DEM1	ILMN_1788180	RAB13
ILMN_3250257	ACVRL1	ILMN_1717477	PSD3

ILMN_1656378	NMT2	ILMN_2359287	ITGA6
ILMN_2225595	ACAD8	ILMN_2375830	DIXDC1
ILMN_1666665	COL23A1	ILMN_2364072	CLCNKA
ILMN_1682781	TEAD2	ILMN_1718128	PABPC3
ILMN_1680856	MAMLD1	ILMN_1695530	MS4A3
ILMN_2317923	TMEM132A	ILMN_3240524	MFSD6
ILMN_1885771		ILMN_1680805	IL28RA
ILMN_1691339	CLEC1A	ILMN_1758895	CTSK
ILMN_1784553	SDC2	ILMN_1751793	PCNXL2
ILMN_2083469	IRS2	ILMN_2123450	FLJ43093
ILMN_2200917	SLC4A7	ILMN_1838767	
ILMN_1740875	FPR2	ILMN_2413780	SEZ6L2
ILMN_2195482	CACNB3	ILMN_1733402	CSF1
ILMN_1685397	ITGA3	ILMN_2305225	NDRG4
ILMN_1785405	SLC17A9	ILMN_1773389	PLTP
ILMN_1699631	GATS	ILMN_1723971	SLC29A1
ILMN_1741014	SLC28A3	ILMN_3236135	FAM86D
ILMN_2398572	FXYD2	ILMN_1684755	KAZALD1
ILMN_2261076	NEDD9	ILMN_2364674	TRPT1
ILMN_3197097	TSTD1	ILMN_1807825	LY86
ILMN_1701483	SYP	ILMN_1754538	C10orf58
ILMN_2128770	CDR2L	ILMN_1724941	CDCP1
ILMN_1786444	LPL	ILMN_1692742	DENND3
ILMN_2404625	LAT	ILMN_1673892	GK5
ILMN_2201678	FSTL1	ILMN_1770161	BST1
ILMN_2100287	CCDC147	ILMN_1690099	ITGB1BP1
ILMN_1765459	S100A13	ILMN_2338963	SLC29A1
ILMN_2387860	CYP19A1	ILMN_2309180	SMARCD3
ILMN_1797585	MYO1B	ILMN_1813572	IL16
ILMN_1775486	SSPN	ILMN_2398939	MBP
ILMN_1749792	SORBS1	ILMN_1777342	PREX1
ILMN_2150856	SERPINB2	ILMN_2117171	LMO4
ILMN_1685403	MMP7	ILMN_1746819	C5
ILMN_2405324	IL28RA	ILMN_1684210	NPAL3
ILMN_1808325	TM4SF19	ILMN_1699139	CYP19A1
ILMN_1730622	EVL	ILMN_1710209	MFSD6
ILMN_1668910	CIDEB	ILMN_3235964	HSPA7
ILMN_3179620	LOC100129673	ILMN_1770641	KLHL3
ILMN_1781045	FXYD2	ILMN_1793730	TM7SF4
ILMN_1867119		ILMN_2052335	MYOC
ILMN_1691539	LAT	ILMN_2372915	P2RY2
ILMN_2192072	MMP7	ILMN_1683755	SOX13
ILMN_1738147	NES	ILMN_1766446	C6orf48
ILMN_2148913	TMEM45A	ILMN_1685009	ITGAM
ILMN_2413644	TM4SF19		

Table A3: CD137L-DC DEG which are ≥ 2x fold-change compared to immature cDCs

Probe_ID	Gene_Symbol	Probe_ID	Gene_Symbol
ILMN_2171384	CXCL5	ILMN_1699139	CYP19A1
ILMN_1752562	CXCL5	ILMN_1733402	CSF1
ILMN_1656310	INDO	ILMN_1808707	FSCN1
ILMN_2192072	MMP7	ILMN_1730906	FILIP1L
ILMN_3239965	IDO1	ILMN_2225595	ACAD8
ILMN_1780465	CLEC5A	ILMN_1699631	GATS
ILMN_1685403	MMP7	ILMN_2359287	ITGA6
ILMN_2065773	SCG5	ILMN_1751228	C20orf46
ILMN_1715131	CCR7	ILMN_1673892	GK5
ILMN_2124221	CLLU1OS	ILMN_1711439	EMILIN1
ILMN_1802653	EBI3	ILMN_1813338	LAG3
ILMN_1789502	GPC4	ILMN_1806249	IL1RN
ILMN_1767113	AOX1	ILMN_1687857	ST6GALNAC4
ILMN_2317923	TMEM132A	ILMN_3205353	LOC100131294
ILMN_2380163	PTPRF	ILMN_2086105	SPRY4
ILMN_1704376	GLDN	ILMN_1682993	NKG7
ILMN_2098446	PMAIP1	ILMN_1709937	KCNN4
ILMN_1785380	SLC1A2	ILMN_1814022	NR1H3
ILMN_2413779	SEZ6L2	ILMN_1675191	GAPT
ILMN_1740875	FPR2	ILMN_2391150	FILIP1L
ILMN_2093027	MYO1B	ILMN_2150851	SERPINB2
ILMN_1769926	DBN1	ILMN_3242271	GAPT
ILMN_2404625	LAT	ILMN_1688480	CCND1
ILMN_1670490	PDPN	ILMN_2049766	NFE2L3
ILMN_2201678	FSTL1	ILMN_1765274	CAPN11
ILMN_1770338	TM4SF1	ILMN_1807925	GNG2
ILMN_1769299	MTMR11	ILMN_1793476	PRKCDBP
ILMN_1681644	BIRC3	ILMN_2395214	FMNL3
ILMN_1669362	IGFBP6	ILMN_2212909	MELK
ILMN_1752965	GREM1	ILMN_1805371	HNRPM
ILMN_1815057	PDGFRB	ILMN_1685703	ACOX2
ILMN_1691539	LAT	ILMN_1784005	LOC644615
ILMN_1811736	ENTHD1	ILMN_1794386	IL2RG
ILMN_3250257	ACVRL1	ILMN_1867119	
ILMN_2374683	PTPN13	ILMN_1680805	IL28RA
ILMN_2289593	FXYD2	ILMN_1774806	SLC9A7
ILMN_1785170	ARMCX2	ILMN_1758827	RTN4IP1
ILMN_1776181	BIRC3	ILMN_1810267	DNHD1
ILMN_1699695	TNFRSF21	ILMN_1805930	CSF1
ILMN_3248591	LTBP2	ILMN_3248352	CCDC88C
ILMN_3251672	DSG2	ILMN_1707124	TFPI
ILMN_1775486	SSPN	ILMN_1804568	HOMER1

ILMN_1698218	TRAF1	ILMN_1662390	ASPHD1
ILMN_1680110	C10orf116	ILMN_2148913	TMEM45A
ILMN_2398572	FXYD2	ILMN_2413064	ST6GALNAC4
ILMN_1762713	C19orf59	ILMN_1726624	YPEL4
ILMN_2170814	LAMP3	ILMN_1742853	LOC652184
ILMN_1695290	FERMT2	ILMN_1679979	PLK3
ILMN_1660806	CSRP2	ILMN_1736127	KIAA0773
ILMN_1805807	SLC30A3	ILMN_1670302	HK3
ILMN_1686555	FYN	ILMN_1701052	TUBG2
ILMN_1808226	RGS16	ILMN_1800787	RFTN1
ILMN_2229261	SIGLEC16	ILMN_1747759	WSB1
ILMN_2316236	HOPX	ILMN_2347592	NMB
ILMN_1740418	CYP27B1	ILMN_1662619	TFPI
ILMN_1690170	CRABP2	ILMN_1786444	LPL
ILMN_2170813	LAMP3	ILMN_1674050	COL8A2
ILMN_1680856	MAMLD1	ILMN_2375830	DIXDC1
ILMN_1781867	FOXD4L1	ILMN_1703123	AXUD1
ILMN_1784948	SPOCD1	ILMN_2102330	COL8A2
ILMN_2068104	TFPI2	ILMN_1788180	RAB13
ILMN_1779965	AK1	ILMN_1786326	KCTD15
ILMN_3245630	ECSCR	ILMN_1751793	PCNXL2
ILMN_2405324	IL28RA	ILMN_1732151	COL6A1
ILMN_1678841	UBD	ILMN_1781791	PRRG1
ILMN_1784532	COL22A1	ILMN_1715068	AQP9
ILMN_3289508	LOC339192	ILMN_1774874	IL1RN
ILMN_1735231	ZSWIM4	ILMN_1794803	NDP
ILMN_1757604	TPM2	ILMN_1898723	
ILMN_2374687	PTPN13	ILMN_1743275	SH3RF3
ILMN_1731374	CPE	ILMN_1669523	FOS
ILMN_2064725	METTL7B	ILMN_2375992	SPINT1
ILMN_2195482	CACNB3	ILMN_1704656	PPP2R1B
ILMN_1914249		ILMN_1731783	ATP1A1
ILMN_1658926	NOTCH3	ILMN_1736704	DIXDC1
ILMN_1781045	FXYD2	ILMN_1655614	DSP
ILMN_1717262	PROCR	ILMN_2160476	CCL22
ILMN_1717727	LOC284293	ILMN_2077733	C12orf30
ILMN_2205963	C10orf54	ILMN_1803988	MCL1
ILMN_1815690	TIE1	ILMN_2357577	PRKAA1
ILMN_1707591	TNIP3	ILMN_1796682	PARP3
ILMN_1656011	RGS1	ILMN_1756992	MUC1
ILMN_1682099	TNFAIP8L3	ILMN_1783156	LOC650832
ILMN_1696031	C15orf21	ILMN_1800276	RCN1
ILMN_1678904	ENO3	ILMN_1693513	LOC644143
ILMN_3248091	C6orf223	ILMN_1788810	C12orf30
ILMN_2124585	GREM1	ILMN_2364072	CLCNKA
ILMN_1748538	ALDH1A2	ILMN_1670638	PITPNC1

ILMN_2380801	FYN	ILMN_1741014	SLC28A3
ILMN_1780831	SLC6A12	ILMN_1666819	PHLDB1
ILMN_2261076	NEDD9	ILMN_1703487	LMO4
ILMN_1773389	PLTP	ILMN_2413780	SEZ6L2
ILMN_2387860	CYP19A1	ILMN_1805916	NIPSNAP1
ILMN_2329927	ABCG1	ILMN_2397954	PARP3
ILMN_2227248	SLAMF9	ILMN_1691736	ST6GALNAC6
ILMN_1682015	GAL	ILMN_2316386	GPBAR1
ILMN_1739001	TACSTD2	ILMN_1717677	ZFHX2
ILMN_1811470	PLEK2	ILMN_1775566	ATP1A1
ILMN_1745623	EFCAB4A	ILMN_1770911	NRSN2
ILMN_1697220	NT5E	ILMN_1805228	LRG1
ILMN_1698666	CST6	ILMN_2355168	MGST1
ILMN_1781207	FYN	ILMN_2119774	CYP2R1
ILMN_1789196	TPM2	ILMN_3302919	MYOF
ILMN_2100437	HBB	ILMN_2148469	RASL11B
ILMN_3197097	TSTD1	ILMN_1684755	KAZALD1
ILMN_1704353	IGSF3	ILMN_2092441	LRP12
ILMN_2143685	CLDN7	ILMN_2305225	NDRG4
ILMN_1749792	SORBS1	ILMN_1653026	PLAC8
ILMN_1800412	BMP1	ILMN_1670870	ALCAM
ILMN_1809859	PCGF2	ILMN_1705297	MYBPH
ILMN_1689525	PMAIP1	ILMN_1682399	CLOCK
ILMN_1656904	SLC1A4	ILMN_1728799	FBP1
ILMN_1806434	PAQR5	ILMN_1678423	SPA17
ILMN_1769615	FLRT2	ILMN_2123450	FLJ43093
ILMN_2112256	TNFRSF4	ILMN_1692742	DENND3
ILMN_2383707	ALDH1A2	ILMN_1810289	FER1L3
ILMN_1794782	ABCG1	ILMN_1680139	MAFF
ILMN_1783996	DEM1	ILMN_1797822	SEL1L3
ILMN_1682781	TEAD2	ILMN_1756572	COQ2
ILMN_1765459	S100A13	ILMN_1787657	CLDN12
ILMN_2365383	ENO3	ILMN_3236135	FAM86D
ILMN_2090802	TMEM79	ILMN_1770641	KLHL3
ILMN_1666665	COL23A1	ILMN_2117171	LMO4
ILMN_2100287	CCDC147	ILMN_2364674	TRPT1
ILMN_1716006	C18orf54	ILMN_1793241	SRD5A1
ILMN_1666546	DUSP14	ILMN_1672743	ZNF334
ILMN_1738578	FILIP1L	ILMN_1677814	ABCC3
ILMN_2349393	MDK	ILMN_1816342	
ILMN_1797585	MYO1B	ILMN_1724612	SLC25A35
ILMN_3298511	LOC728537	ILMN_2413508	CD97
ILMN_1724699	ACAD8	ILMN_1747673	RASL10A
ILMN_1670535	NDRG2	ILMN_1662741	EDG4
ILMN_1701483	SYP	ILMN_1798533	ZNF22
ILMN_2150856	SERPINB2	ILMN_2370976	FER1L3

ILMN_1738147	NES	ILMN_1788387	UGCGL2
ILMN_1744381	SERPINE1	ILMN_1701643	GDPD5
ILMN_1750181	TESC	ILMN_1747395	SLC24A1
ILMN_1659106	PHLDA3	ILMN_1770922	TMEM45A
ILMN_2361603	NDRG2	ILMN_1656378	NMT2
ILMN_1746819	C5	ILMN_2394161	ST8SIA4
ILMN_1677441	C20orf123	ILMN_1760890	SEPN1
ILMN_1885771		ILMN_1694400	MSR1
ILMN_1666902	GPR114		

Table A4: CD137L-DC DEG which are ≥ 2x fold-change compared to macrophages

Probe_ID	Gene_Symbol	Probe_ID	Gene_Symbol
ILMN_2160476	CCL22	ILMN_1797822	SEL1L3
ILMN_1656310	INDO	ILMN_1808325	TM4SF19
ILMN_2170814	LAMP3	ILMN_1722738	ROGDI
ILMN_3239965	IDO1	ILMN_1694400	MSR1
ILMN_1739001	TACSTD2	ILMN_1709937	KCNN4
ILMN_2192072	MMP7	ILMN_2073604	EBP
ILMN_1789502	GPC4	ILMN_1724946	SPINT1
ILMN_1740418	CYP27B1	ILMN_2355168	MGST1
ILMN_1786444	LPL	ILMN_1721623	APOO
ILMN_1710186	CCL17	ILMN_1700047	ALAS1
ILMN_1685403	MMP7	ILMN_1810267	DNHD1
ILMN_1715131	CCR7	ILMN_2261076	NEDD9
ILMN_1794803	NDP	ILMN_1680659	C11orf63
ILMN_1867119		ILMN_1689842	SC4MOL
ILMN_2361603	NDRG2	ILMN_1795507	ABCA6
ILMN_1784948	SPOCD1	ILMN_1800787	RFTN1
ILMN_1802653	EBI3	ILMN_1677765	LRP8
ILMN_1688480	CCND1	ILMN_2395214	FMNL3
ILMN_1911721		ILMN_2413644	TM4SF19
ILMN_1748538	ALDH1A2	ILMN_1807177	KIAA1797
ILMN_1785380	SLC1A2	ILMN_1686555	FYN
ILMN_2124221	CLLU1OS	ILMN_1783996	DEM1
ILMN_1744381	SERPINE1	ILMN_1760890	SEPN1
ILMN_1752562	CXCL5	ILMN_3181411	ATL1
ILMN_1659106	PHLDA3	ILMN_2134974	RAB38
ILMN_1717477	PSD3	ILMN_2399392	SIL1
ILMN_2319000	MATK	ILMN_2399036	SEPN1
ILMN_2317923	TMEM132A	ILMN_2347592	NMB
ILMN_1733535	ZNF366	ILMN_1813338	LAG3
ILMN_2227248	SLAMF9	ILMN_1815626	DHCR7

ILMN_2201678	FSTL1	ILMN_1680139	MAFF
ILMN_1769926	DBN1	ILMN_1788387	UGCGL2
ILMN_2383707	ALDH1A2	ILMN_1704369	LIMA1
ILMN_2332250	ACOT7	ILMN_3205353	LOC100131294
ILMN_1776519	RAP1GAP	ILMN_1814022	NR1H3
ILMN_2098446	PMAIP1	ILMN_1784871	FASN
ILMN_1815190	METTL1	ILMN_1781207	FYN
ILMN_1698218	TRAF1	ILMN_1663538	CLYBL
ILMN_1682015	GAL	ILMN_1715068	AQP9
ILMN_1705686	NRGN	ILMN_1735231	ZSWIM4
ILMN_1776181	BIRC3	ILMN_2413508	CD97
ILMN_1685703	ACOX2	ILMN_1720889	SC4MOL
ILMN_1678841	UBD	ILMN_2090802	TMEM79
ILMN_1770338	TM4SF1	ILMN_1745623	EFCAB4A
ILMN_3251672	DSG2	ILMN_1701643	GDPD5
ILMN_1805930	CSF1	ILMN_1805916	NIPSNAP1
ILMN_1659352	KCNK13	ILMN_1784005	LOC644615
ILMN_1747673	RASL10A	ILMN_2077733	C12orf30
ILMN_2405324	IL28RA	ILMN_1915188	
ILMN_1712352	DOCK3	ILMN_2413527	VCL
ILMN_2171384	CXCL5	ILMN_2380801	FYN
ILMN_1735157	GALNT12	ILMN_1786326	KCTD15
ILMN_3306997	METTL1	ILMN_1675542	LOC729148
ILMN_2112256	TNFRSF4	ILMN_1719649	TMEM63A
ILMN_3289508	LOC339192	ILMN_1772241	SQLE
ILMN_1666546	DUSP14	ILMN_1690040	TM7SF2
ILMN_1781867	FOXD4L1	ILMN_1716006	C18orf54
ILMN_1784553	SDC2	ILMN_1678729	SIL1
ILMN_1660806	CSRP2	ILMN_1755281	FBXO15
ILMN_1733402	CSF1	ILMN_1885771	
ILMN_1789196	TPM2	ILMN_2041293	SQLE
ILMN_2374449	SPP1	ILMN_1767253	RRP12
ILMN_2289593	FXYD2	ILMN_1756806	MCL1
ILMN_1780465	CLEC5A	ILMN_2165867	DHCR7
ILMN_3298511	LOC728537	ILMN_1759453	UQCRB
ILMN_1770641	KLHL3	ILMN_1726624	YPEL4
ILMN_2370976	FER1L3	ILMN_1786658	BOLA3
ILMN_2047618	KCNE1	ILMN_1804248	FDPS
ILMN_1670302	HK3	ILMN_1657395	HMGCR
ILMN_1704353	IGSF3	ILMN_1712088	CLYBL
ILMN_3248091	C6orf223	ILMN_2381121	UQCC
ILMN_1656011	RGS1	ILMN_1684210	NPAL3
ILMN_1651354	SPP1	ILMN_1667199	SQRDL
ILMN_1772821	KIAA1671	ILMN_1788180	RAB13
ILMN_1719627	SLC27A3	ILMN_2224103	PAPSS1
ILMN_2398572	FXYD2	ILMN_1663751	CYCSL1

ILMN_1769299	MTMR11	ILMN_3250257	ACVRL1
ILMN_1656904	SLC1A4	ILMN_1803988	MCL1
ILMN_2375992	SPINT1	ILMN_2225595	ACAD8
ILMN_1763638	BCAR3	ILMN_1758750	EARS2
ILMN_1696031	C15orf21	ILMN_1693513	LOC644143
ILMN_3302919	MYOF	ILMN_2401927	TTC8
ILMN_1810289	FER1L3	ILMN_1703228	AGFG2
ILMN_1717262	PROCR	ILMN_1724699	ACAD8
ILMN_1658926	NOTCH3	ILMN_2373285	MSR1
ILMN_1725510	DHCR24	ILMN_3234116	LOC730382
ILMN_1793476	PRKCDBP	ILMN_1759023	WFS1
ILMN_1779965	AK1	ILMN_1703123	AXUD1
ILMN_1773389	PLTP	ILMN_1705849	SPR
ILMN_1652237	CBR3	ILMN_1768558	LOC727848
ILMN_1679460	PPFIBP1	ILMN_1781819	PAPSS1
ILMN_2374865	ATF3	ILMN_1736539	ALDH1L2
ILMN_1781045	FXYD2	ILMN_1791306	C9orf103
ILMN_1743199	EGR2	ILMN_1718621	TSPAN32
ILMN_1655100	LOC644680	ILMN_1657955	FMNL3
ILMN_1742853	LOC652184	ILMN_3248591	LTBP2
ILMN_1717727	LOC284293	ILMN_3307791	FAM69A
ILMN_1670870	ALCAM	ILMN_1758281	CALCRL
ILMN_1678904	ENO3	ILMN_1657550	MVD
ILMN_1680856	MAMLD1	ILMN_1717677	ZFHX2
ILMN_1771261	SYNC1	ILMN_2397954	PARP3
ILMN_2365383	ENO3	ILMN_1770466	ATP5G3
ILMN_2391150	FILIP1L	ILMN_2382648	MID2
ILMN_1724941	CDCP1	ILMN_1741096	FDFT1
ILMN_3235176	C2orf89	ILMN_1699737	TRAP1
ILMN_2229261	SIGLEC16	ILMN_1729167	EFTUD1
ILMN_1713679	MREG	ILMN_1683273	SNAPC5
ILMN_1704084	CMAH	ILMN_2069128	EPB41L2
ILMN_1775486	SSPN	ILMN_1712291	MICALL2
ILMN_1655796	Mar-03	ILMN_2373763	CASP7
ILMN_1732151	COL6A1	ILMN_1742547	NRP1
ILMN_1689525	PMAIP1	ILMN_2049536	TRPV2
ILMN_2407824	ATP1B1	ILMN_1782635	YARS2
ILMN_1806249	IL1RN	ILMN_1671568	ECHDC2
ILMN_2188722	GLS	ILMN_1782015	FCRLB
ILMN_2316386	GPBAR1	ILMN_1737580	PPPDE2
ILMN_1788778	Sep-11	ILMN_1745152	UQCC
ILMN_1660871	NEK6	ILMN_1724497	ABI2
ILMN_1688152	IL27RA	ILMN_3217276	LOC644517
ILMN_2340347	PC	ILMN_1662741	EDG4
ILMN_1685397	ITGA3	ILMN_1803945	HCP5
ILMN_1804568	HOMER1	ILMN_2123450	FLJ43093

ILMN_1704656	PPP2R1B	ILMN_1770031	ABHD10
ILMN_2349393	MDK	ILMN_3236135	FAM86D
ILMN_1774806	SLC9A7	ILMN_1793241	SRD5A1
ILMN_2143685	CLDN7	ILMN_1803392	TAX1BP3

Appendix III: Tissue culture media

Media	Constituent	Amount
RPMI 1640 (1 L preparation)	RPMI powder (Sigma-Aldrich)	16.35 g
	200 mM L-glutamine (Gibco, Invitrogen)	10 ml
	Sodium Bicarbonate (Sigma-Aldrich)	2.0 g
	MilliQ water	Top to 1L
R10 PS (1 L preparation)	RPMI 1640 medium	890 ml
	FBS (Biowest)	100 ml
	Penicillin/Streptomycin (Gibco, Invitrogen)	10 ml
R10 2Ab (1 L preparation)	RPMI 1640 medium	980 ml
	Human Ab serum (Sigma-Aldrich)	20 ml
R10 PS PM (1 L preparation)	R10 PS media	997 ml
	1M Probenecid solution in NaOH	2 ml
	50 mM β-mercaptoethanol stock	1 ml
AIMV 2Ab (1 L preparation)	AIM V® Medium, containing L-glutamine, streptomycin sulphate at 50 µg/ml, and gentamicin sulphate at 10 µg/ml (Gibco, Invitrogen)	980 ml
	Human Ab serum (Sigma-Aldrich)	20 ml
AIMV 2Ab PM (1 L preparation)	AIMV 2Ab medium	997 ml
	1M Probenecid solution in NaOH	2 ml
	50 mM β-mercaptoethanol stock	1 ml

The pH of all culture media was adjusted to 7.4 and sterile filtered through a 0.22 µm filter.

Appendix IV: Buffers and reagents

Buffer	Constituent	Amount
PBS + 2 mM EDTA (1 L preparation)	10 × PBS (1st Base)	100 ml
	0.5 M EDTA (1st Base)	4 ml
	MilliQ water	Top to 1L
RBC lysis buffer (1 L preparation)	NH_4Cl (Sigma-Aldrich)	8.29 g
	$NaHCO_3$ (Sigma-Aldrich)	0.84 g
	0.5 M EDTA (1st Base)	23 μl
	MilliQ water	Top to 1L
MACS buffer (1 L preparation)	10 × PBS (1st Base)	100 ml
	0.5 M EDTA (1st Base)	4 ml
	BSA (Biowest)	5 g
	MilliQ water	Top to 1L
Staining buffer (1 L preparation)	FBS	5 ml
	Sodium azide (Sigma-Aldrich)	0.2 g
	10 × PBS (1st Base)	100 ml
	MilliQ water	Top to 1L
Trypsin-EDTA (50 ml preparation)	0.5 % Trypsin-EDTA (Gibco, Invitrogen)	5 ml
	10 × PBS (1st Base)	5 ml
	MilliQ water	Top to 50 ml
10 mM EDTA in PBS (50 ml preparation)	0.5 M EDTA (1st Base)	1 ml
	10 × PBS (1st Base)	5 ml
	MilliQ water	Top to 50 ml

The pH of all buffers was adjusted to 7.4 and sterile filtered through a 0.22 μm filter (except for staining buffer).

Appendix V: Publications

International Scientific Publication(s)

1. Harfuddin Z, Kwajah S, Chong Nyi Sim A, MacAry PA, Schwarz H. CD137L-stimulated dendritic cells are more potent than conventional dendritic cells at eliciting cytotoxic T-cell responses. OncoImmunology 2013; 2:e26859

2. Tang Q, Jiang D, Harfuddin Z, Cheng CK, Moh MC, Schwarz H. Regulation of myelopoiesis by CD137 ligand signalling. (In preparation)

3. Harfuddin Z, Duan K, Poidinger M, Koh LK, Wong SC, Schwarz H. Transcriptional regulation in CD137 ligand generated dendritic cells. (In preparation)

Selected Local & International Scientific Conference Posters & Oral Presentation

1. Harfuddin Z, Kwajah S, Schwarz H. Activation of antigen-specific T lymphocytes by CD137 ligand generated dendritic cells. Keystone Symposia: Dendritic cells and the Initiation of Adaptive Immunity. Santa Fe, New Mexico, USA, February 2011. (Poster Presentation).

2. Harfuddin Z, Kwajah S, Chong Nyi Sim A, MacAry PA, Schwarz H. Activation of antigen-specific T lymphocytes by CD137 ligand generated dendritic cells. European Macrophage and Dendritic Cell Society. Debrecen, Hungary, September 2012. (Poster Presentation).

3. Harfuddin Z, Kwajah S, Chong Nyi Sim A, MacAry PA, Schwarz H. Activation of antigen-specific T lymphocytes by CD137 ligand generated dendritic cells. Frontiers in Immunology and Inflammation: From Molecules to Disease. Tokyo, Japan, February 2013. (Poster Presentation and was presented with a travel award).

4. Harfuddin Z, Kwajah S, Chong Nyi Sim A, MacAry PA, Schwarz H. Activation of antigen-specific T lymphocytes by CD137 ligand generated dendritic cells. 6th International Singapore Symposium of Immunology. Singapore, June 2013. (Poster Presentation).

5. Harfuddin Z, Kwajah S, Chong Nyi Sim A, MacAry PA, Schwarz H. CD137 ligand generated dendritic cells are more potent than classical dendritic cells in inducing an antigen-specific T cell response. 15th International Congress of Immunology. Milan, Italy, August 2013. (Poster Presentation).

6. Harfuddin Z, Duan K, Poidinger M, Koh LK, Wong SC, Schwarz H. Transcriptional and functional characterization of dendritic cell differentiation induced by CD137 ligand reverse signalling in human monocytes. 2nd International Graduate Student Immunology Congress. Marseille, France, August 2013. (Selected for Oral Presentation).